UNIVERSIDADE FEDERAL DO PARANÁ

GILLIZE APARECIDA TELLES DE ARAUJO

CARACTERIZAÇÃO *IN VITRO* DAS ENZIMAS MÁLICA MaeB E N-ACETIL GLUTAMATO QUINASE NAGK E INTERAÇÃO COM PROTEÍNAS PII EM *Azospirillum brasilense*

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Orientador: Prof. Dr. Luciano Fernandes Huergo
Co-orientadora: Dra. Edileusa Cristina Marques Gerhardt

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RESUMO

As proteínas PII são conhecidas por regular o metabolismo de nitrogênio e carbono em bactérias, algas e plantas. Ensaios preliminares utilizando a proteobactéria diazotrófica *Azospirillum brasilense* mostraram que a proteína PII GlnZ deste organismo pode interagir com a enzima málica $NADP^+$ dependente (MaeB1) e com a enzima N-acetil glutamato quinase (NAGK). MaeB1 está envolvida na conversão de malato a piruvato, com redução de $NADP^+$ a NADPH. Essa enzima possui uma extensão C-terminal extra, homóloga às fosfotransacetilases (PTA). NAGK atua na biossíntese de arginina, e em muitos organismos é inibida por este aminoácido. Vários estudos demonstram que NAGKs de cianobactérias, algas e plantas, que são sensíveis à arginina, são capazes de interagir com PII. Para validar estes ensaios preliminares, nós realizamos ensaios de co-precipitação das enzimas em questão com as proteínas PII de *A. brasilense.* Nossos resultados mostram que GlnZ interage com AbMaeB e AbNAGK *in vitro*, em condições de alta concentração de nitrogênio celular. GlnZ parece regular a atividade málica de AbMaeB a fim de fornecer mais NADPH para as reações biossintéticas. Ensaios cinéticos visando a caracterização da atividade de enzima málica de AbMaeB1 foram realizados, AbMaeB1 é fortemente ativada por concentrações fisiologicamente relevantes de glutamina Este efeito também foi observado para a enzima ortóloga de *E. coli* (EcMaeB). Nós também demonstramos pela primeira vez que AbMaeB e EcMaeB possuem domínio PTA ativo capaz de converter coenzima A e acetil-fosfato em Acetil-CoA.

Palavras-chave: Proteínas PII, interação, MaeB, NAGK

ABSTRACT

The PII are signal transduction proteins that are known to regulate the nitrogen and carbon metabolism, in bacteria, plants and algae. Preliminary assays using the diazotrophic Proteobacterium *Azospirillum brasilense* showed that the PII protein GlnZ interacts with the malic enzyme (AbMaeB1) and with the N-acetyl glutamate kinase (NAGK). The AbMaeB1 converts malate to pyruvate, with reduction of $NADP^+$ to NADPH. This enzyme also carries a C-terminal extension, which shows homology with phophotransacetylases (PTA). NAGK acts in the arginine biosynthesis and in many organisms is feed-back inhibited by this aminoacid. Several studies showed that NAGKs from cyanobacteria, algae and plants are arginine-sensitive and can interact with PII to relieve such feedback inhibition. In this work we have performed co-purification assays to show that both MaeB1 and NAGK interact with the PII proteins in *A. brasilense*. Our data show that GlnZ forms a complex with AbMaeB and AbNAGK in condition of high intracellular nitrogen concentrations. GlnZ may be regulating the direct malic activity in order to provide NADPH to nitrogen assimilation. Kinetic analysis of the malic activity of AbMaeB1 indicated strong activation by physiological relevant glutamine concentrations. The same effect was detected in the *E. coli* orthologue enzyme (EcMaeB). We also demonstrated for the first time that the PTA domain of AbMaeB and EcMaeB is active and that they are activated by glutamine on a dose-dependent manner**.**

Keywords: PII proteins, interaction, MaeB, NAGK

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LISTA DE ABREVIATURAS

2-OG	2-Oxoglutarato
ACC	Acetil-CoA Carboxilase
AcK	Acetato quinase
ADP	5’ difosfato de adenosina
AMPPNP	Adenilil-imidodifosfato
Amp^r	Resistência a ampicilina
ATP	5’ trifosfato de adenosina
BC	Biotina carboxilase
BCCP	Proteína carreadora de carboxi biotina
BSA	Albumina bovina
DNA	Ácido desoxirribonucleico
DO_{600}	Densidade óptica a 600 nm
DraG	Dinitrogenase redutase glicohidrolase
DraT	Dinitrogenase ADP-ribosil transferase
DTT	Ditiotreitol
GlnZ-UMP	Proteína GlnZ uridililada
GOGAT	Glutamato sintase
GS	Glutamina sintetase
IPTG	Isopropil β-D-1- tiogalactopiranosideo
Kb	Kilobases
Km^r	Resistência a canamicina
LC/MS	Cromatografia líquida acoplada a espectrometria de massa
MALDI-TOF	*Matrix-Assisted laser desorption*
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
NAD^+	Dinucleotideo de nicotinamida oxidado
NADH	Dinucleotideo de nicotinamida reduzido
$NADP^+$	Fosfato dinucleotideo de nicotinamida oxidado
NADPH	Fosfato dinucleotídeo de nicotinamida reduzido
NAG	N-acetil glutamato
NAGK	Acetil glutamato quinase
NAGS	N-acetil glutamato sintase
Ntr	Sistema de regulação de nitrogênio

OAA	Oxaloacetato
PCK	Fosfoenolpiruvato carboxiquinase
PCR	Reação em cadeia de polimerase
PEP	Fosfoenolpiruvato
PGPB	Bactérias promotoras do crescimento vegetal
Pi	Fosfato inorgânico
Ppsa	Fosfoenolpiruvato sintetase
PTA	Fosfotransacetilase
PTS	Sistema de fosfotransferases
Rpm	Rotações por minuto
SDS	Dodecil sulfato de sódio
SDS-PAGE	Eletroforese em géis de poliacrilamida
TCA	Ciclo dos ácidos tricarboxílicos
UMP	Uridina monofosfato
UR	Removedor de uridilil
UT	Função de uridililação
UTP	Uridina trifosfato
UV	Ultravioleta
V/V	Volume por volume

SUMÁRIO

1. INTRODUÇÃO

1.1. *Azospirillum* spp. e fixação de nitrogênio

As bactérias do gênero *Azospirillum* pertencem à classe das α proteobactérias (YOUNG, 1992), são diazotróficas, ou seja, capazes de fixar o nitrogênio atmosférico (N_2), se associam à raízes de diversas plantas de interesse agrícola (DÖBEREINER e DAY, 1976: DÖBEREINER, 1991; ECKERT *et al*., 2001; STEENHOUDT; VANDEREYDEN, 2000), são microaeróbicas, gram negativas, curvas, móveis (ECKERT *et al.,* 2001), de vida livre e fixadoras de nitrogênio. Dezoito espécies do gênero já foram descritas, sendo *A. brasilense* a mais estudada (ALMEIDA, 2016).

São consideradas bactérias promotoras do crescimento vegetal (PGPB), pois são capazes de melhorar o crescimento de muitas espécies de plantas, de importância ecológica e agronômica (BASHAN e DE-BASHAN, 2010; DOBBELAERE *et al.* 2001). O uso de *Azospirillum* como inoculante é promissor, muitos estudos detalhando os efeitos benéficos da inoculação com estes microrganismos foram realizados e descrevem mudanças fisiológicas e morfológicas nas plantas inoculadas. Ainda assim, em muitos casos o processo e os compostos que induzem estas respostas não são claramente identificados (BASHAN; HOLGUIN; DE-BASHAN, 2004). Durante as últimas décadas, a inoculação de diversas plantações com espécies de *Azospirillum* tem sido intensamente estudada, evidenciando seus benefícios ao melhorar o crescimento vegetal, aumentando a produtividade (BASHAN; HOLGUIN; DE-BASHAN, 2004). Pesquisadores do estado do Paraná testaram e selecionaram estirpes de *Azospirillum* que melhor sobreviviam no solo, se adaptavam às tecnologias empregadas em milho e trigo e promoviam maior crescimento da planta. Estes estudos foram promissores para a autorização pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA) de diversas estirpes de *A. brasilense* na produção de inoculantes comerciais para a cultura do milho e trigo. Os estudos demonstraram que há incremento médio de 24% a 30% no rendimento de grãos de milho quando inoculados com *A. brasilense* e de 13 a 18% para trigo (HUNGRIA *et al.*, 2010).

Este aumento no rendimento provavelmente é devido ao fato de *A. brasilens*e produzir fitormônios, como auxinas (PRINSEN *et al.,* 1993), citocininas (TIEN *et al*., 1979), giberilinas (BOTTINI *et al*., 1989), etileno (STRZELCZYK *et al*., 1994), e outros reguladores do crescimento que melhoram o crescimento da raiz, adsorção de água e minerais

(DOBBELAERE *et al.,* 2001), além da habilidade destes microrganismos de fixar nitrogênio. A teoria mais aceita é de que o efeito benéfico da associação com *Azospirillum* ocorra devido a um efeito sinérgico de múltiplos mecanismos (BASHAN; HOLGUIN; LUZ, 2004).

A fixação de nitrogênio pela bactéria *A. brasilense* representa um alto gasto energético, pois utiliza um complexo proteíco (complexo da nitrogenase) para converter o gás dinitrogênio atmosférico (N_2) a amônio, com um gasto de 16 moléculas de ATP por molécula de N_2 fixado. Por conta disso, as vias metabólicas relacionadas a fixação e metabolismo de nitrogênio são finamente reguladas tanto em sua atividade, quanto no nível de expressão gênica (POSTGATE, 1982). Uma das peças chave na regulação e coordenação de várias proteínas relacionadas ao metabolismo do nitrogênio são as proteínas da família PII.

1.2. Proteínas PII

As proteínas PII são conhecidas por regular a atividade de uma ampla variedade de enzimas, fatores de transcrição e transportadores transmembrana através da interação direta proteína-proteína (HUERGO; CHANDRA e MERRICK, 2012; MERRICK, 2015). Os alvos conhecidos estão em sua grande maioria envolvidos em algum aspecto do metabolismo celular de nitrogênio (HUERGO; CHANDRA e MERRICK, 2012). Membros desta superfamília de proteínas ocorrem em quase todas as bactérias, em arqueas e em cloroplastos de algas vermelhas e plantas (ARCONDÉGUY *et al.,* 2001), constituindo desta forma a maior família de proteínas sinalizadoras na natureza (FORCHAMMER e LÜDDECK, 2016). Arcondéguy e colaboradores (2001) subdivide as proteínas PII em 3 grupos principais: as codificadas pelos genes *glnB, glnK* ou *nifI*. Em sua pesquisa Sant'anna (2009) ainda adiciona um quarto grupo, as chamadas PII New Group (PII-NG), proteínas que não possuem alguns sítios característicos de PII, mas que claramente são relacionadas a elas pela homologia das sequências.

Em sua maioria, proteobactérias e arqueas codificam múltiplas proteínas PII (geralmente GlnB e GlnK), enquanto cianobactérias e plantas codificam apenas uma (MERRICK, 2015). As proteínas GlnB são distribuídas majoritariamente entre as proteobactérias, enquanto as GlnK são distribuídas de forma mais ampla. Algumas proteínas, como GlnZ de *A. brasiliense* possuem uma relação filogenética com GlnK, apresentam função similar, mas não são ligadas ao gene *amtB* (SANT'ANNA *et al.,* 2009).

Em todos estes grupos em que estão presentes, as proteínas PII apresentam estrutura conservada, são proteínas triméricas, com massa de 12-13 kDa por monômero, possuem

formato cilíndrico e cada monômero é composto por 2 α hélices e 4 folhas β (HUERGO; CHANDRA e MERRICK, 2012), sendo que cada subunidade contém três estruturas em volta (*loops*): *loop* T, B e C. O *loop* T fica exposto ao solvente e é o ponto chave para a maioria das interações entre as proteínas PII e seus diversos alvos, pois facilita a interação por conta de sua flexibilidade (ARCONDEGUY *et al,* 2001). O *loop* T e B de um monômero e o *loop* C do monômero adjacente formam uma fenda lateral que constitui um sítio de ligação (HUERGO; CHANDRA e MERRICK, 2012), onde ADP, ATP e 2-oxoglutarato (2-OG), as chamadas moléculas efetoras, podem se ligar (Figura 1) (TRUAN *et al.,* 2010). A ligação destas moléculas à proteína PII é uma característica conservada em quase todos os membros da superfamília (FORCHAMMER e LÜDDECK, 2016) e está relacionada a movimentos moleculares na proteína PII que influenciam na conformação do *loop* T e consequentemente na habilidade de interagir com seus alvos (CONROY *et al.,* 2007; TRUAN *et al.,* 2010). A ligação de ATP e ADP ocorre de forma competitiva (JIANG *et al*, 2007), e a ligação de 2-OG e ATP de forma cooperativa (KAMBEROV et al., 1995, JIANG *et al.,* 2007), e requer o envolvimento do cátio divalente Mg^{2+} (XU *et al.,* 1998). Além disso, ADP age antagonisticamente a 2-OG (JIANG e NINFA, 2007).

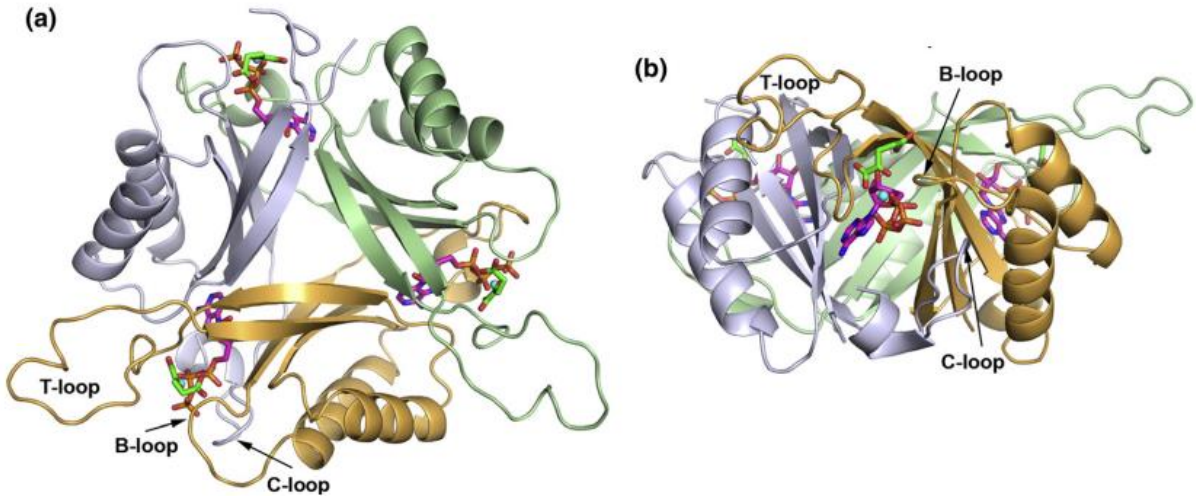

Figura 1 Representação do trímero de GlnZ de *A. brasilense*. O trímero está ligado à ATP (magenta), 2-OG (verde) e Mg^{2+} (azul). Os *loops* B, C e T são indicados por setas. A) Vista superior. B) Vista lateral. Fonte: TRUAN *et al.,* 2010.

1.2.1. As proteínas PII e metabólitos chave

A ligação das moléculas efetoras ATP, ADP e 2-OG, bem como os níveis de glutamina intracelular caracterizam os dois modos de percepção de sinal pelas proteínas PII (FORCHHAMMER, 2008; NINFA e JIANG, 2005; JIANG e NINFA, 2007).

O 2-OG serve como esqueleto carbônico para a síntese de aminoácidos e sua concentração intracelular pode sinalizar os níveis de nitrogênio (HUERGO *et al.,* 2012) e de carbono (ZHANG, WEI e YE, 2013; ZHANG e YE, 2014), ATP tem papel essencial como fornecedor de energia para as reações enzimáticas, sinalizando o estado energético da célula. Dessa forma, devido à capacidade de PII ligar tanto ATP, ADP e 2-OG elas agem como sensoras do estado energético da célula, através da relação ATP/ADP (Gerhardt, 2012) e sensoras dos níveis de carbono e nitrogênio. Apesar disso, na alga verde *Chlamydomonas reinhardtii* e no musgo *Physcomitrella patens* não é observada ligação da proteína PII à ADP, mostrando que em algum momento na evolução houve uma diferenciação no sensoriamento de energia, com uma especialização na ligação de ATP. PII de *P. patens* também não responde à 2-OG (LAPINA *et al.,* 2018).

Vários trabalhos mostram que as concentrações de 2-OG e a proporção ATP/ADP influenciam a formação do complexo GlnK-AmtB em *E. coli* (DURAND e MERRICK, 2006; TEIXEIRA *et al.,* 2008; WOLFE, ZHANG e ROBERTS, 2007; CONROY *et al.,* 2007). Variações nos níveis de ATP/ADP *in vitro* também influenciam os complexos entre NtrB e ATase com GlnB em *E.coli* (JIANG E NINFA, 2009), entre GlnB e NAGK e PipX em *Synecchococus elongatus* (FOKINA *et al.,* 2011), e entre DraG e AmtB e GlnZ e AmtB e GlnB em *A. brasilense* (GERHARDT *et al.,* 2012). Gerhardt *et al.* (2012) demonstram que na interação entre GlnZ-DraG e GlnZ-AmtB *in vitro* em *A. brasilense*, a ligação de ATP ou ADP à proteína PII vai depender da concentração de 2-OG, em altas concentrações de 2-OG, a ligação de ATP é favorecida, enquanto em baixas concentrações a ligação de ADP é favorecida, o que demonstra o importante papel de 2-OG como sinalizador para as proteínas PII.

Vários estudos demonstram a variação desses metabólitos dentro da célula em diferentes condições de nitrogênio. Radchenko, Thornton e Merrick (2010) determinaram que os níveis intracelulares de 2-OG variaram de 1,4 mM para 0,3 mM depois de 2 min de choque de amônio. Os níveis de ATP e ADP sofreram alteração após 30 segundos, mas logo se reestabeleceram, dessa forma, os níveis de ATP/ADP parecem ser tamponados (BENNETT *et al.,* 2009; CHAPMAN, FALL e ATKINSON, 1971; RADCHENKO, THORNTON e MERRICK, 2010). No ensaio de Gerhardt (2012) a proporção ATP/ADP também praticamente não mudou com a variação de nitrogênio em células crescidas em condições de limitação deste composto, já a concentração de 2-OG passou de 4,5 a 1,7 mM depois do choque de amônio.

Em geral, os estudos que medem os níveis de ATP/ADP após choque de amônio variam desde uma rápida queda no nível de ATP (SCHUTT e HOLZER, 1972) a nenhuma flutuação (YUAN *et al*., 2009; ZHANG, POHLMANN e ROBERTS, 2009). Os métodos de amostragem,

condições de cultura e técnicas utilizadas para medir influenciam o resultado (SCHNEIDER e GOURSE, 2003; RADCHENKO, THORNTON e MERRICK, 2010).

Além de responder a efetores, o comportamento das proteínas PII também é influenciado por glutamina, metabólito de central importância na fisiologia microbiana, pois é o produto primário da assimilação de nitrogênio, doador de nitrogênio em várias reações biossintéticas e um dos 20 aminoácidos necessários para síntese de proteínas (FORCHHAMMER, 2007).

Em proteobactérias, a glutamina é utilizada como molécula sinalizadora chave. O sinal de glutamina é transmitido para a proteína PII de forma indireta, a glutamina regula a uridililação reversível de PII em Proteobacteria (FORCHHAMMER, 2007). Em algumas plantas e algas, a glutamina pode se ligar diretamente às proteínas PII (CHELLAMUTHU *et al*., 2014), tendo papel chave na interação das proteínas PII e proteínas alvo.

Estas propriedades tornam as proteínas PII capazes de sensoriar mudanças nos níveis celulares de ATP e ADP (tornando-as sensoras do estado energético da célula), além de integrar as informações da relação ATP/ADP com os níveis intracelulares de 2-OG (FORCHAMMER e LÜDDECK, 2016) e glutamina (FORCHHAMMER, 2007).

1.2.2. Importância do 2-OG como molécula sinalizadora

A molécula de 2-OG além de ser intermediária no ciclo TCA também serve como esqueleto carbônico para a assimilação de nitrogênio, atuando nas vias de síntese do glutamato (COMMICHAU, FORCHAMMER, STULKE, 2006; CHUBUKOV e SAUER, 2014), dessa forma os níveis desta molécula variam de acordo com a disponibilidade de amônio (HUERGO e DIXON, 2015). Radchenko, Thorthon e Merrick (2010), mostram em *E. coli* que os níveis de 2-OG diminuem após choque de amônio, e mostram que há uma correlação inversa entre a disponibilidade de amônio e acúmulo de 2-OG. Há evidências de que os níveis intracelulares de 2-OG também sinalizam a disponibilidade de carbono em *E. coli.* Os trabalhos de Zhang, Wei e Ye (2013) e Zhang e Ye (2014) mostram que há um aumento significativo nos níveis de 2-OG quando glucose é adicionada às culturas de *E. coli*. Por conta disso, a molécula de 2-OG age como sinalizador metabólico, refletindo o balanço de carbono e nitrogênio na célula - os dois principais nutrientes necessários pelos organismos vivos - e regula muitas proteínas e vias metabólicas como os sistemas PTS^{Glu}, PTS^{Ntr}, a proteína adenilato ciclase e as proteínas PII, que parecem ser uma das proteínas mais ancestrais capazes de sensoriar 2-OG (HUERGO E

DIXON, 2015). Desta forma, as proteínas PII conseguem integrar o estado de carbono e nitrogênio celular pois ligam diretamente 2-OG.

Apesar de sua importância, a ligação de 2-OG não é universal. A proteína GlnK de *Archaeoglobus fulgidus* e PII de *P. patens*, por exemplo, não conseguem ligar esta molécula (HELFMANN *et al.,* 2010; LAPINA *et al.,* 2018). No caso de *A. fulgidus* pode significar que GlnK responda apenas a variações energéticas e não ao níveis de nitrogênio (HELFMANN et al, 2010). No caso de *P. patens* esta falta de sensibilidade ao 2-OG pode ser contornada pela presença do *loop* Q, que liga diretamente a glutamina (LAPINA *et al.,* 2018).

1.2.3. Modificações pós traducionais em proteínas PII

Além da regulação alostérica, algumas proteínas PII ainda têm uma segunda resposta sensorial relacionada à modificação pós traducional (HUERGO; CHANDRA e MERRICK, 2012). Em proteobactérias, estas modificações ocorrem no resíduo de tirosina-51 do *loop* T de cada monômero por modificação covalente, onde um grupamento uridilil pode ser adicionado (SON, RHEE, 1986; FORCHHAMMER, 2004), a modificação é realizada pela enzima GlnD (uridililtransferase/removedora de uridilil - UTase/UR), codificada pelo gene *glnD*. A enzima GlnD é considerada sensora dos níveis de nitrogênio intracelular (MERRICK E EDWARDS, 1995), pois possui um sítio de ligação de glutamina, e a concentração deste metabólito responde à disponibilidade de nitrogênio (MERRICK, 2014).

Quando o nitrogênio disponível na forma de amônio é baixo, há uma redução nos níveis intracelulares de glutamina, e a enzima GlnD catalisa a uridililação da PII. Por outro lado, quando o nitrogênio disponível na forma de amônio é alto, ocorre um aumento nos níveis de glutamina, este metabólito se liga à enzima GlnD que por sua vez promove a remoção do grupo uridilil de PII (ENGLEMAN E FRANCIS, 1978). A uridililação/desudirilação ocorrem na presença de ATP e 2-OG e a uridililação responde à concentração celular de glutamina, sendo inibida por ela (ENGLEMAN e FRANCIS, 1978; ARAUJO *et al*, 2008). As PII podem ser encontradas como trímeros livres, mono (PII-UMP_1), di (PII-UMP_2) ou triuridililados (PIIUMP_3) e essa modificação é importante para direcionar a interação com suas proteínas alvos, como DraT, DraG, AmtB, NtrB e NifA (HUERGO *et al*., 2006, 2007 e 2009; VAN DOMMELLEN *et al*., 2002; ZHU *et al*., 2006).

Além da modificação por uridililação, alguns organismos sofrem outros tipos de modificação no *loop* T. Estudos de modificação pós traducional da PII em duas actinobactérias,

Streptomyces coelicolor e *Corynebacterium glutamicum,* mostraram que nestes organismos o papel de GlnD é adenililar PII, ao invés de uridililá-la (HESKETH *et al.,* 2002; STROSSER *et al.*, 2004). As modificações ocorrem também no resíduo de tirosina na posição 51 do *loop* T (MERRICK, 2014).

A fosforilação é uma outra forma de modificação da PII que ocorre em cianobactéria, no resíduo de serina na posição 49 do *loop* T e também ocorre em resposta à limitação de nitrogênio (MERRICK, 2014). Em *Anabaena* sp. ainda ocorre nitração na tirosina-51 (ZHANG *et al.,* 2007).

Em vários grupos taxonômicos, as proteínas PII não sofrem qualquer forma de modificação pós traducional (SANT'ANNA, 2009). Na maioria das plantas não há necessidade de uma modificação no *loop* T, pois a glutamina se liga diretamente na PII, isso ocorre por meio de uma modificação na cauda C-terminal, onde há a formação de duas pequenas alfa hélices que são conectadas por um loop de 5 aminoácidos, denominado *loop* Q, onde a glutamina se liga. Esta estrutura é desordenada na ausência de glutamina e quando a glutamina está presente ela se forma por meio de pontes de hidrogênio, faz contato com o *loop* T e transmite o sinal de glutamina (CHELLAMUTHU *et al*., 2014).

1.2.4. Proteínas PII na regulação da fixação biológica de nitrogênio

O processo de fixação do nitrogênio é bem estudado em proteobactérias e as proteínas PII desempenham papeis complexos em sua regulação. O modo primário de regulação é pelo controle da transcrição dos genes de fixação de nitrogênio (genes *nif*) e isso pode ser alcançado regulando a expressão e a atividade de reguladores transcricionais (ARCONDÉGUY, 2001). Neste contexto, algumas interações já bem caracterizadas são entre as proteínas PII e NtrB, NifA, DraT e DraG de vários organismos diazotróficos.

PII e NtrB

Em geral, a regulação do metabolismo do nitrogênio é controlado a nível transcricional pelo sistema de regulação de nitrogênio (ntr) que regula a utilização de fontes alternativas de nitrogênio (ZHANG *et al.,* 1997). Em *E. coli,* este sistema envolve pelo menos sete proteínas: NtrB, NtrC, GlnD, GlnB, GlnK, GlnE e glutamina sintetase (GlnA) (MERRICK e EDWARDS, 1995), envolvidas em uma cascata regulatória onde a proteína PII é o elemento central, pois

controla a atividade das outras proteínas dependendo dos níveis de nitrogênio intracelular (NINFA e ATKINSON, 2000; ARCONDÉGUY, JACK e MERRICK, 2001).

As proteínas NtrB e NtrC fazem parte de um sistema de dois componentes que respondem aos níveis de nitrogênio (HANKENBECK e STOCK, 1996). Quando os níveis de nitrogênio são altos, glutamina intracelular aumenta, GlnD desuridilila GlnB (ARCONDÉGUY, JACK e MERRICK, 2001), que se liga à NtrB e desfosforila NtrC, inativando-a (JIANG e NINFA, 1999). Quando os níveis de glutamina são baixos, GlnD adiciona um grupamento uridilil à GlnB (ARCONDÉGUY, JACK e MERRICK, 2001), GlnB não consegue se ligar à NtrB, que ativa NtrC por fosforilação, NtrC por sua vez ativa a transcrição de genes envolvidos na assimilação de fontes alternativas de nitrogênio (ATKINSON *et al*., 1994; JIANG e NINFA, 1999).

NifA e PII

Em proteobactérias diazotróficas, a transcrição dos genes *nif* é dependente dos promotores de σ^{54} em conjunto com a proteína NifA, que atua como reguladora da transcrição (DIXON, 1998; MERRICK, 1993). O controle da transcrição dos genes *nif* é regulado pela expressão ou atividade da NifA em resposta a oxigênio ou a nitrogênio fixado (DIXON E KAHN, 2004). Quase todas as proteínas NifA possuem 3 domínios funcionalmente diferentes (DRUMMOND, WHITTY e WOOTTON, 1986) na porção N terminal está o domínio GAF, que controla sua interação com outras proteínas (DRUMMOND *et al.,* 1990).

Em diazotrofos pertencentes às α proteobactérias e algumas β proteobactérias, a atividade de NifA é regulada diretamente por PII (DIXON E KAHN, 2004). Em *A. brasilense*, *Herbaspirillum seropedicae* e *Rhodospirillum rubrum* as proteínas PII são necessárias para ativar NifA quando nitrogênio é limitante, enquanto em *Rhodobacter capsulatus* e *Gluconacetobacter diazotrophicus* as PII parecem ser necessárias para prevenir a atividade de NifA quando o nitrogênio está abundante.

Em *A. brasilense,* NifA é inibida por seu domínio GAF. Quando há limitação de nitrogênio, a proteína GlnB é uridililada e passa a interagir com NifA, permitindo a ativação dos genes *nif* (ARSENE *et al.,* 1999; VAN DOMMELEN *et al*., 2002; HUERGO *et al.,* 2005). Esse mecanismo permite que a expressão dos genes *nif* responda a flutuações na disponibilidade de amônio, neste organismo (CHEN *et al.,* 2005; HUERGO *et al*., 2005).

DraT/DraG e PII

A regulação da atividade da nitrogenase também é regulada a nível pós-traducional, algumas α proteobactérias diazotróficas, incluindo *A. brasilense* podem regular a atividade da nitrogenase por ADP ribosilação em resposta a amônia ou limitação de energia (HARTMANN e BURRIS, 1986; LIANG *et al.,* 1992; LUDDEN, 1994; MASEPOHL, KREY e KLIPP, 1993; ZHANG *et al.,* 1997).

Essa é uma reação catalisada pelas enzimas DraT (dinitrogenase redutase ADP-ribosil transferase) e DraG (dinitrogenase redutase glicohidrolase). DraT promove adição de um grupamento ribosil à arginina 101 da subunidade da dinitrogenase redutase, inativando-a. DraG remove o grupo ribosil, reativando a enzima (LUDDEN, 1994).

As duas enzimas estão sujeitas à regulação oposta pelas proteínas PII (ZHANG *et al.,* 1997; NORDLUND, 2000). Em *A. brasilense*, GlnB interage diretamente com DraT (HUERGO *et al.,* 2006a, 2009), e GlnZ com DraG (HUERGO *et al.,* 2007, 2009). Em condições de choque de amônio a GlnB é desuridililada e interage com DraT, ativando-a e levando à ADP ribosilação de NifH, que é então inativada (MOURE *et al*, 2013), nas mesmas condições GlnZ também é desuridililada e o complexo GlnZ-DraG se liga à membrana, formando um complexo ternário com AmtB (HUERGO *et al.,* 2006b), levando a uma inativação de DraG (HUERGO *et al.,* 2007).

1.2.5. Participação da PII além do metabolismo de nitrogênio

Por conta da molécula de 2-OG ter amplo papel dentro do metabolismo, surgiu a hipótese de que as proteínas PII pudessem coordenar também o metabolismo de carbono (NINFA, JIANG, 2005; COMMICHAU, FORCHHAMMER, STÜLKE, 2006). Esta hipótese foi confirmada em 2010, quando Feria Bourrelier e colaboradores mostraram que a subunidade BCCP da enzima Acetil CoA carboxilase (ACC) – enzima chave que catalisa a primeira reação da biossíntese de ácidos graxos - de *Arabidopsis thaliana* é alvo da proteína PII, esta foi a primeira enzima descrita como alvo de PII que não está envolvida no metabolismo de nitrogênio. Nos anos seguintes, estudos identificaram por meio de abordagem proteômica, que a subunidade BCCP de ACC de *A. brasilense* interage com PII e que GlnK de *E. coli* forma um complexo estável com BCCP (RODRIGUES *et al*, 2014, GERHARDT *et al*, 2015), também foi mostrado que a proteína GlnB destes dois organismos são capazes de formar um complexo ternário com as subunidades BC-BCCP de ACC (GERHARDT *et al,* 2015). A interação observada em organismos distantes evolutivamente sugere que é uma interação conservada e

que BCCP é um ligante ancestral de PII (RODRIGUES *et al,* 2014), a funcionalidade do complexo também parece ser conservado, pois em *A. thaliana* e *E. coli* o complexo parece inibir a atividade de ACC (FERIA BOURRELLIER *et al*., 2010; HUERGO E DIXON, 2015).

Depois destas descobertas, Gerhardt (2015) realizou ensaios a fim de encontrar outros potenciais alvos da proteína PII em *A. brasilense.* Foram então realizados 3 ensaios de interação da proteína His-GlnZ com extrato de *A. brasiliense* 2812 (mutante duplo *glnBglnZ*), utilizando condições diferenciais de ATP/ADP e 2-OG para interação e eluição, visto a importância destes efetores para interação. As frações obtidas nestes ensaios foram analisadas por espectrometria de massa quantitativa sem marcação (LC/MS/MS) e foram determinadas as proteínas diferencialmente expressas entre GlnZ e controle, a partir destas foram selecionadas as que estavam presentes pelo menos 15 vezes mais do que no controle, o que resultou em uma lista de 125 proteínas possíveis alvos de GlnZ, que estão envolvidas no metabolismo de carboidratos, lipídeos, aminoácidos, cofatores e vitaminas, nucleotídeos, metabolismo do nitrogênio, proteínas chaperonas, fatores de transcrição, mobilidade celular, processamento e reparo de DNA, resistência a drogas, transdução de sinal e transporte de membrana, além de proteínas não classificadas (GERHARDT, 2015).

Dentre os potenciais alvos, estão a acetilglutamato quinase (NAGK) e a enzima málica $NADP^+$ dependente (maeB; EC 1.1.1.40) que são os alvos do presente estudo.

1.3. N-acetilglutamato quinase (NAGK)

Cianobactérias e plantas estocam nitrogênio na forma de arginina, que é incorporada nas proteínas (VAN ETTEN *et al*., 1963; SLOCUM, 2005) ou em polímeros ricos em arginina (MAHESWARAN *et al.,* 2006), estando também relacionadas com a síntese de poliaminas, importantes para o crescimento celular (LEE *et al.,* 2009). A arginina é ideal para estoque de nitrogênio, pois sua incorporação nas proteínas minimiza seu impacto osmótico e o nitrogênio é facilmente mobilizado (QUINTERO *et al.,* 2000).

N-acetilglutamato quinase (NAGK; E.C. 2.7.2.8) é a segunda enzima da biossíntese de arginina, e promove a fosforilação do grupamento –COOH do N-acetilglutamato (NAG) formando N-acetilglutamil fosfato na segunda etapa da biossíntese de arginina (CUNIN *et al*., 1986) (ATP + N-acetilglutamato = ADP + N-acetilglutamil 5-fosfato) e sofre ativação por seu substrato, glutamato (MCKAY e SHARGOOL, 1981).

Em muitos organismos, como leveduras, algas, plantas e a maioria das bactérias, a atividade de NAGK é um ponto crucial de regulação, sendo retroinibida pelo produto final, arginina (CUNIN *et al.,* 1986). Apesar disso, em muitos organismos ela é insensível à arginina (FERNÁNDEZ-MURGA *et al.,* 2004). Em *E. coli*, por exemplo, é a primeira enzima da rota, N-acetilglutamato sintase (NAGS) quem sofre inibição pelo produto final (FERNÁNDEZ MURGA e RUBIO, 2008).

A diferença na sensibilidade por arginina está em uma extensão N-terminal de 16-25 resíduos, presentes exclusivamente nas NAGKs sensíveis à arginina (FERNÁNDEZ-MURGA *et al.,* 2004; RAMÓN-MAIQUES *et al.,* 2002; RAMÓN-MAIQUES *et al.,* 2006) e formam uma hélice, denominada N-hélice. Por meio desta cauda, três homodímeros se ligam, formando um hexâmero (RAMÓN-MAIQUES *et al.,* 2006), enquanto as NAGKs não sensíveis à arginina são diméricas, como a de *E. coli* (Figura 2) (FERNANDÉZ-MURGA, 2008; SLOCUM, 2005).

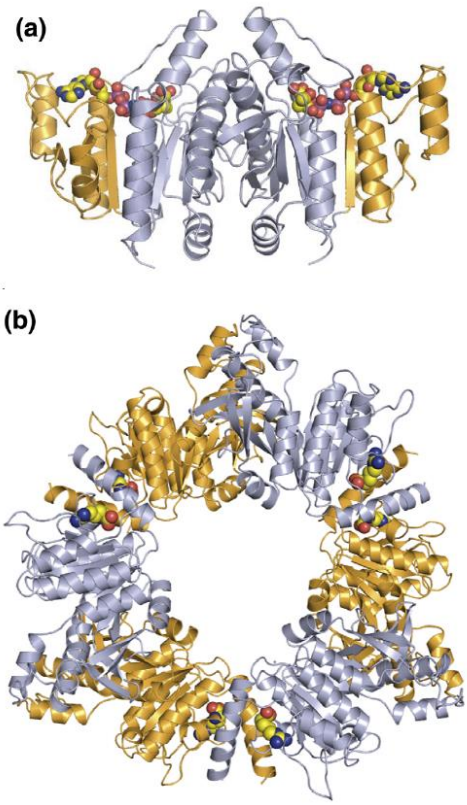

Figura 2 - A) Representação do dímero de NAGK insensível à arginina de *E. coli*, ligada à AMPPNP (análogo ao ATP) e NAG. Em cinza domínios C-terminais e em amarelo domínios N-terminais de cada subunidade. B) Representação do hexâmero de NAGK sensível à arginina de *Thermotoga marítima* ligado à arginina. Três subunidades são representadas em cinza e as outras três em amarelo. Fonte: Adaptado de LLÁCER, FITA e RUBIO, 2008.

A organização hexamérica e a presença dessas hélices são essenciais para fazer da NAGK uma reguladora da rota, inibida por arginina, exibindo uma cinética sigmoidal de inibição por este composto (MAHESWARAN, URBANKE e FORCHHAMMER, 2004;

FERNÁNDEZ-MURGA *et al*., 2004; FERNÁNDEZ-MURGA e RUBIO, 2008). Assim, em concentrações de arginina abaixo de um certo limite (10 mM para *S. elongatus* (MAHESWARAN, URBANKE e FORCHHAMMER, 2004), a enzima não é inibida, permitindo a síntese de arginina, porém acima deste limite a enzima é abruptamente inibida, parando a síntese (LLÁCER, FITA e RUBIO, 2008).

A inibição por arginina e ativação por glutamato da NAGK, representam etapas chave na regulação da produção da arginina (SHARGOOL, JAIN e MCKAY, 1988). Uma terceira forma de regulação de NAGK também é de suma importância: a ativação pela proteína PII.

1.3.1. NAGK e interação com PII

Como NAGK é inibida por arginina (SLOCUM, 2005; CUNIN *et al.,* 1986; XU, LABEDAN, GLANSDORFF, 2007), essa enzima deve ser liberada da inibição para conseguir formar estoques deste composto. Em cianobactérias, como *S. elongatus* e em cloroplastos de plantas quem realiza este efeito é a proteína PII.

Na cianobactéria *S. elongatus*, a proteína PII, quando não fosforilada e em condições de excesso de nitrogênio, se liga à NAGK, liberando-a da inibição e permitindo a síntese de arginina (HEINRICH *et al.,* 2004). Quando nitrogênio está escasso, 2-OG acumula e liga PII na presença de ATP, promovendo a dissociação do complexo PII-NAGK (MAHESWARAN, URBANKE e FORCHHAMMER, 2004). A ligação de ADP à PII e a fosforilação promovida por 2-OG previnem a formação do complexo em cianobactérias (HEINRICH *et al.,* 2004; BURILLO *et al.,* 2004; MAHESWARAN, URBANKE e FORCHHAMMER, 2004). Não se sabe se a ligação da PII diminui a sensibilidade à arginina por bloquear os sítios de ligação ou por induzir mudanças conformacionais na enzima. Burillo *et al.* (2004) demonstraram uma regulação similar em *A. thaliana* e sugeriram que esse mecanismo pode ser conservado em cianobactérias e cloroplastos. A regulação pela PII desta enzima chave na biossíntese de arginina, pode ser uma forma de PII ter um controle mais fino no metabolismo do nitrogênio (LLÁCER, FITA e RUBIO, 2008).

O complexo consiste de dois trímeros de PII envolvendo um hexâmero (trímero de dímeros) de NAGK, cada subunidade de PII contata uma subunidade oposta de NAGK e envolve principalmente os *loops* B e T de PII (Figura 3) (LLÁCER *et al*., 2007). Cada subunidade da PII interage com uma subunidade de NAGK, longe dos sítios de arginina e de substratos, o *loop* T fica na forma compacta, por isso, quando ADP se liga, favorecendo a forma

estendida do *loop*, o complexo é dissociado em *S. elongatus* (MAHESWARAN, URBANKE e FORCHHAMMER, 2004).

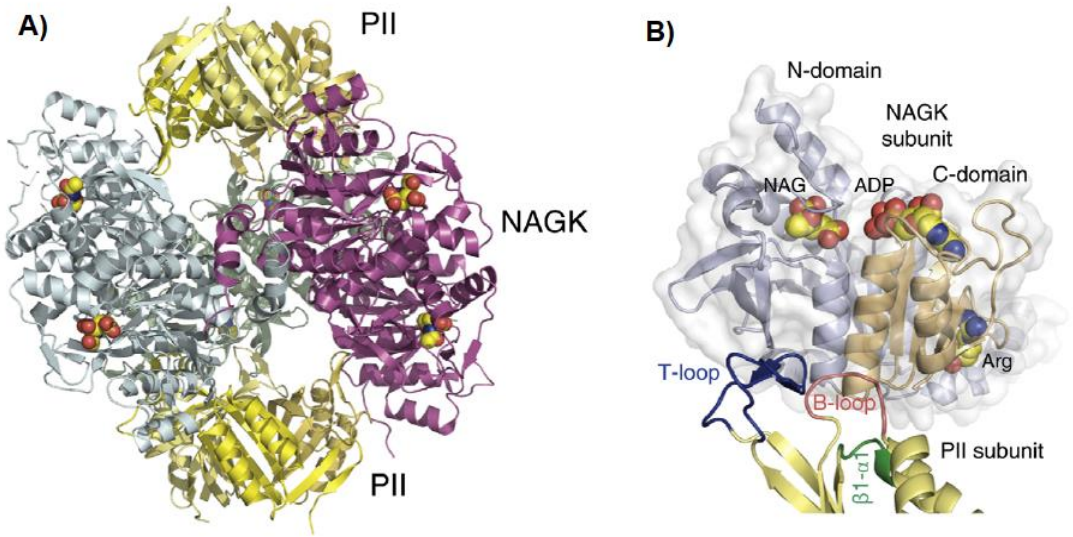

Figura 3 - Complexo entre PII e NAGK de *S. elongatus*. A) Hexâmero de NAGK ligado à PII, os dímeros são representados pelas cores cinza claro, cinza escuro e roxo, os trímeros de PII estão representados em amarelo. B) Envolvimento dos *loops* B e T na formação do complexo NAGK-PII. Fonte: Adaptado de LLÁCER, FITA e RUBIO, 2008.

As NAGKs de cianobactérias e plantas, apesar de bastante similares, apresentam algumas diferenças funcionais e também diferenças substanciais nos complexos com proteínas PII (LLÁCER, FITA e RUBIO, 2008). Uma diferença aparente entre a proteína PII de *A. thaliana* (AtPII) e a PII de *S. elongatus* (SePII) é a resposta a ADP na formação do complexo. Enquanto ADP inibe fortemente a formação do complexo SePII-NAGK, a ligação de AtPII com AtNAGK não é eficientemente antagonizada por esta molécula, a interação parece ser tão forte que continua mesmo em altas concentrações de ADP. Além disso, na maioria das plantas superiores a interação NAGK-PII é afetada diretamente por glutamina, através do *loop* Q presente nestes organismos (CHELLAMUTHU *et al.,* 2014). Desta forma, plantas e cianobactérias podem ter desenvolvido diferentes estratégias para sensoriar e integrar os sinais de energia para regulação da interação PII-NAGK (MAHESWARAN, URBANKE e FORCHHAMMER, 2004).

Apesar destas diferenças, Beez *et al.* (2009) mostraram que SePII é capaz de interagir com AtNAGK, este estudo demonstrou que PII de bactérias e eucariotos podem pelo menos em parte ser funcionalmente permutáveis. Considerando mais de 1,2 bilhões de anos separando cianobactérias de plantas (SHEVELEVA e HALLICK, 2004), a conservação desta interação pode ser explicada por uma alta pressão seletiva para manter a interação PII-NAGK. Assim,

controlar a bissíntese de arginina em organismos fototróficos através da energia (sensoriada por ATP/ADP) e carbono/nitrogênio (sensoriado por 2-OG) deve ser de fundamental importância (FERRARIO-MERY *et al.,* 2006).

É importante ressaltar que todas as interações até então evidenciadas ocorrem em organismos que possuem NAGK hexamérica e sensível à arginina, já em *E. coli,* a NAGK se apresenta na forma dimérica, sendo insensível à arginina e não há evidência de interação até agora. Desta forma, Maheswaran e colaboradores (2004) sugerem que a forma hexamérica seria o ponto chave para a interação entre NAGK e PII. Em *A. brasiliense* não há informação sobre o mecanismo de NAGK e não se sabe se ela apresenta-se na forma dimérica ou hexamérica, entretanto ela apresenta maior similaridade com NAGK de *S. elongatus* (52% de identidade e ~70% de similaridade) que com a de *E. coli* (32% de identidade e ~50% de similaridade). Além disso, dos 5 resíduos necessários para a interação com PII (LLÁCER *et al.,* 2007), dois estão presentes na NAGK de *A. brasiliense,* enquanto nenhum é encontrado na enzima de *E. coli*, o que sugere que NAGK de *A. brasiliense* é mais similar a de cianobactérias, o que aumenta a possibilidade de interação com PII (GERHARDT, 2015).

1.4. Enzimas málicas

A enzima málica é responsável por catalisar a descarboxilação oxidativa de malato a piruvato e CO_2, bem como a carboxilação redutiva de piruvato (L-malato + $NAD(P)^+ \overset{Me^{2+}}{\rightleftharpoons}$ piruvato + CO_2 + NAD(P)H) (FUKUDA *et al.*, 2005), a primeira reação está associada a redução de NAD(P)+ a NAD(P)H (CHANG; TONG, 2003). O malato formado pelas enzimas málicas, pode ser utilizado para repor intermediários do ciclo do ácido tricarboxílico (TCA). Alternativamente, quando os intermediários do TCA estão em excesso podem ser direcionados para piruvato e então para outras vias como a gliconeogênese (OWEN *et al.,* 2002). As enzimas málicas fazem parte do importante nó metabólico PEP/piruvato/OAA que liga a glicólise e gliconeogênese com o ciclo TCA (Figura 4).

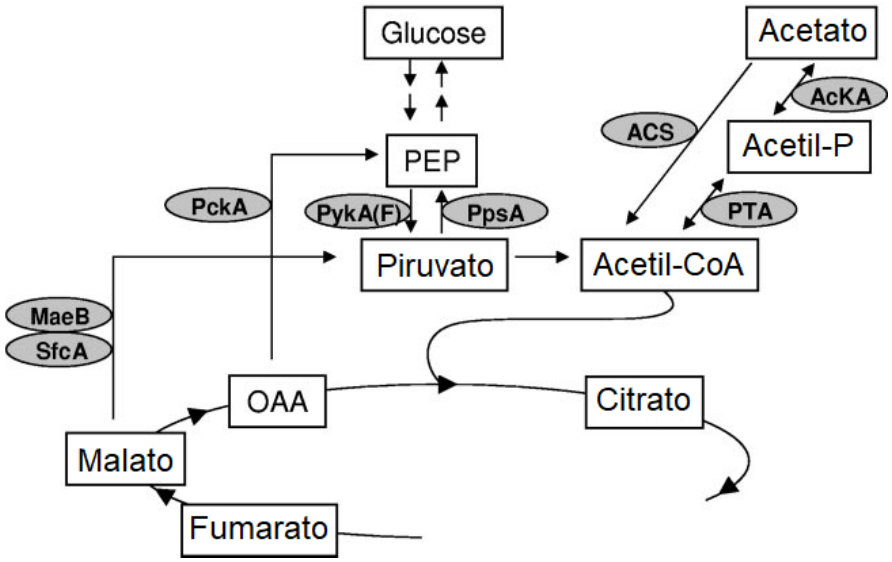

Figura 4 - Nó PEP/piruvato/OAA e sua conexão com glicólise/gliconeogênese. As enzimas málicas (MaeB e/ou SfcA), juntamente com a enzima PEP sintetase (PpsA) formam PEP, o precursor direto da gliconeogênese. Adaptado de BOLOGNA, ANDREO e DRINCOVICH, 2007.

As enzimas málicas possuem papel crucial para crescimento em intermediários do TCA ou em substratos que entram no metabolismo central via acetil-coenzima A (CoA), como é o caso do acetato (BOLOGNA *et al.,* 2007). Estes fatores as tornam importantes dentro das vias anapleróticas, pois são repositoras de compostos do ciclo (FUKUDA *et al.,* 2005).

Estas enzimas são amplamente distribuídas e bastante conservadas, em eucariotos encontram-se as seguintes isoformas: enzima málica $NADP^+$ dependente citosólica (cNADP-ME) (EC 1.1.1.40), enzima málica $NADP^+$ dependente mitocondrial (mNADP-ME) (EC 1.1.1.40) e enzima málica NAD(P)+ dependente mitocondrial (mNAD(P)-ME), com preferência por NAD+ (CHANG; TONG, 2003), em plantas há a isoforma cloroplástica (EDWARDS; ANDREO, 1992), e organismos que apresentam hidrogenossomo podem apresentar a enzima nesta organela (DOLEŽAL *et al.*, 2004). Em procariotos sua classificação leva em consideração além da sua especificidade de cofator também sua capacidade de descarboxilar oxaloacetato (OAA): NAD+ dependentes que descarboxilam OAA (EC 1.1.1.38), NAD dependentes que não descarboxilam OAA (EC 1.1.1.39) e $NADP^+$ dependentes que descarboxilam OAA (EC 1.1.1.40) (BOLOGNA; ANDREO; DRINCOVICH, 2007), apesar de relatos de algumas enzimas málicas $NADP^+$ dependentes não apresentarem a capacidade de descarboxilar OAA (DOLEŽAL *et al.*, 2004; FUKUDA *et al.*, 2005; VOEGELE; MITSCH; FINAN, 1999).

1.4.1. Enzimas málicas procarióticas

Em *Escherichia coli* há dois genes com homologia às enzimas málicas: o gene *sfcA* (ou *maeA*) que cofidica para a enzima málica NAD^+ dependente (SfcA) e o gene *maeB* (ou *ypfF*) cujo produto é a enzima málica $NADP^+$ dependente (MaeB). SfcA é um tetrâmero com subunidades de 64 kDa, enquanto MaeB apresenta-se como octâmero, com subunidades de 83 kDa (BOLOGNA 2007).

Três domínios conservados podem ser observados nas enzimas málicas de procariotos, dois específicos para a atividade: domínio N-terminal e o domínio de ligação de NAD^+/$NADP^+$. Em MaeB de *E. coli* e de algumas outras bactérias gram negativas (BOLOGNA; ANDREO; DRINCOVICH, 2007; MITSCH *et al*., 1998; VOEGELE; MITSCH; FINAN, 1999), é encontrado um domínio extra carboxi terminal, que possui homologia com a enzima fosfotransacetilase (PTA) (Figura 5) (BOLOGNA, ANDREO e DRINCOVICH, 2007).

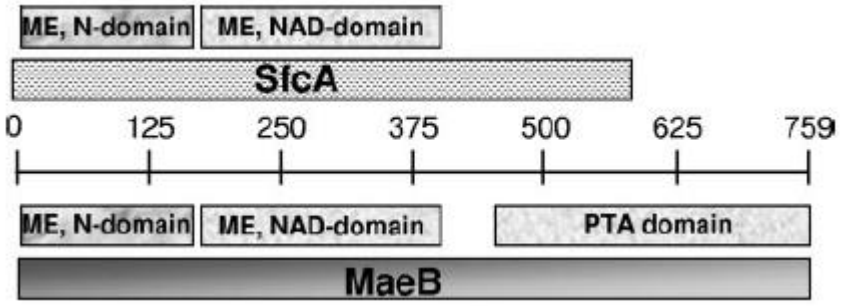

Figura 5 - Representação dos domínios das enzimas málicas SfcA e MaeB de *Escherichia coli.* Dois domínios são conservados na maioria das enzimas málicas, domínio N terminal e domínio NAD+. MaeB possui ainda um domínio adicional, o domínio PTA. Fonte: BOLOGNA, ANDREO e DRINCOVICH, 2007.

A maioria dos procariotos possui enzimas málicas NAD^+ e $NADP^+$ dependentes, que apresentam diferentes funções. Em geral, a enzima málica NAD^+ dependente junto com a piruvato desidrogenase possuem papel na conversão de malato a acetil-CoA (DRISCOLL; FINAN, 1993), Outra função importante, descoberta em 1993 por Driscoll e Finan é o papel na fixação de nitrogênio. Neste estudo, os autores construíram um mutante de *Rhizobium meliloti* que produzia uma enzima málica NAD^+ dependente inativa e o inocularam em plântulas de alfafa, os nódulos foram formados e se desenvolveram como a estirpe selvagem até o momento de iniciar a fixação, mas não apresentaram capacidade de fixação de nitrogênio.

Já a enzima málica $NADP^+$ dependente fornece NADPH para as reações biossintéticas (GOURDON *et al*., 2000). Em resumo, assume-se que as enzimas málicas $NADP^+$ dependentes fornecem o poder redutor para a biossíntese de vários componentes celulares, enquanto as

NAD^+ dependentes promovem a entrada de carbono derivado de aminoácidos ou dicarboxilatos C4 na gliconeogênese (FUKUDA *et al.*, 2005).

Análises de sequências de enzimas málicas eucarióticas e procarióticas mostraram que em procariotos existem sete deleções conservadas, em relação às sequências de eucariotos e que estas deleções são dispersas pelo comprimento da cadeia polipeptídica. Entretanto, nenhum dos resíduos indispensáveis para a atividade de enzimas eucarióticas estão ausentes na estrutura primária de enzimas procarióticas. Apesar destas diferenças, o sítio ativo das enzimas málicas, regiões que contatam subunidades e sítio de ligação a metais são regiões altamente conservadas entre elas (CHANG; TONG, 2003; DOLEŽAL *et al.*, 2004; MITSCH *et al.,* 1998), portanto todas devem compartilhar o mesmo mecanismo catalítico (CHANG; TONG, 2003; DOLEŽAL *et al.,* 2004).

Com relação à cinética, muitas enzimas málicas apresentam controle alostérico de metabólitos chaves. A reação málica direta, catalisada pela MaeB de *E. coli* (L-malato + $NAD(P)^+ \xrightleftharpoons{Me^{2+}}$ piruvato + CO_2 + NAD(P)H) é inibida por acetil-CoA, OAA e fumarato e ativada por glutamato, aspartato, glucose-6-fosfato e acetil fosfato, enquanto SfcA é ativada apenas por aspartato e inibida por acetil-fosfato, CoA, OAA e palmitoil-CoA (BOLOGNA, ANDREO e DRINCOVICH, 2007). Trabalhos anteriores mostraram que a expressão de SfcA e MaeB é induzida durante o metabolismo de acetato em *E. coli* (OH *et al.,* 2002). Duplo mutante *sfcA*/*maeB*, durante o estado de transição entre glucose e acetato, apresentou uma severa fase lag, mesmo possuindo a enzima PEP carboxiquinase (PCK) superexpressa (enzima que forma PEP a partir de OAA em condições gliconeogênicas). Estes dados sugerem que as enzimas málicas possuem um papel além de direcionar o fluxo de carbono para PEP (KAO, TRAN e LIAO, 2005). No trabalho de Bologna, Andreo e Drincovich (2007), regulação alostérica por derivados de acetato foi encontrada em ambas as enzimas málicas de *E. coli*, o caso mais intrigante é o acetil-fosfato, que regula reciprocamente as duas isoformas, acetil-fosfato parece ser um sinalizador global em *E. coli* e bactérias relacionadas, transmitindo informação para vários sistemas de dois componentes (FREDERICKS *et al.,* 2006; MCCLEARY, STOCK e NINFA, 1993), podendo inclusive fosforilar NtrC *in vitro* (FENG *et al.*, 1992). A diferença da influência de vários compostos – entre eles o acetil-P - nas enzimas málicas NAD^+ e $NADP^+$ dependente, parece revelar uma preferência por uma das isoformas em determinadas condições fisiológicas, em concentrações altas de acetil fosfato por exemplo, a isoforma $NADP^+$ dependente possui atividade favorecida, o que pode indicar que esta

molécula poderia mediar um sinal para indução ou repressão da atividade das enzimas málicas de *E. coli*.

1.4.2. Enzimas málicas com domínio PTA

Algumas bactérias gram negativas como *Rhizobium melioti* (MITSCH *et al*., 1998; VOEGELE; MITSCH; FINAN, 1999), *E. coli* (BOLOGNA; ANDREO; DRINCOVICH, 2007), e *A. brasilense* (este trabalho) as enzimas málicas tipo MaeB possuem um domínio extra, de aproximadamente 320 aminoácidos, com homologia às fosfotransacetilases (PTA) na região C-terminal. Em *E. coli,* este domínio C-terminal da MaeB possui 350 aminoácidos e é 31% similar a proteína EutD de *E. coli*, envolvida com a utilização de etanolamina neste organismo e 29% idêntica a proteína PTA do mesmo organismo (BOLOGNA; ANDREO; DRINCOVICH, 2007).

Em *E. coli,* o domínio PTA de MaeB parece estar envolvido na regulação da atividade da enzima málica por acetil-CoA, glutamato, aspartato e glucose-6-fosfato, sugerindo que esses efetores interagem alostericamente com este domínio, regulando a atividade de MaeB. Mutantes sem este domínio apresentam eficiência catalítica da atividade málica 5 vezes menor e não são afetados por esses compostos. Essa cauda também está envolvida na oligomerização, a associação dos dois domínios deve facilitar o controle metabólico de MaeB (BOLOGNA; ANDREO; DRINCOVICH, 2007).

O domínio PTA, visto que está presente em várias bactérias gram negativas, deve possuir relevância funcional, porém a função exata desta cauda extra ainda não é clara, já que até o momento não havia sido encontrada atividade PTA nestas enzimas (BOLOGNA; ANDREO; DRINCOVICH, 2007; MITSCH *et al*., 1998).

1.5. Enzimas fosfotransacetilases (PTA)

Enzimas fosfotransacetilases (PTA; EC 2.3.1.8) convertem acetil-CoA e fosfato inorgânico a acetil-fosfato e CoA (ROSE *et al.,* 1954). Brinsmade e Escalante-Semerena (2007) dividem a proteína PTA em duas classes diferentes: PTA I, que possui aproximadamente 350 aminoácidos de comprimento, e PTA II, que possui aproximadamente 700 aminoácidos de comprimento. Essas duas classes possuem 40% de identidade, sendo que PTA I compartilha

homologia com a porção C-terminal de PTA II (CAMPOS-BERMUDEZ *et al.*, 2010). A enzima PTA de *E. coli* (que é do tipo PTA II) possui três domínios conservados, o P-*loop*, contendo NTPase na cauda N-terminal, para ligação de nucleotídeo fosfato. Este domínio N-terminal parece ser importante para a estabilização da forma hexamérica da PTA II em *E. coli* e na regulação por metabólitos, já que proteínas truncadas sem este domínio não formaram hexâmero e não respondem aos reguladores (CAMPOS-BERMUDEZ *et al.,* 2010). O segundo domínio é o DRTGG, com função desconhecida e por último, um domínio compartilhado pelas fosfato acetil/burtaril transferases (domínio PTA_PTB) no final C-terminal. As PTA I só possuem este último domínio, sendo assim considerado o que está associado com a atividade catalítica da enzima (Figura 6) (CAMPOS-BERMUDEZ *et al.,* 2010).

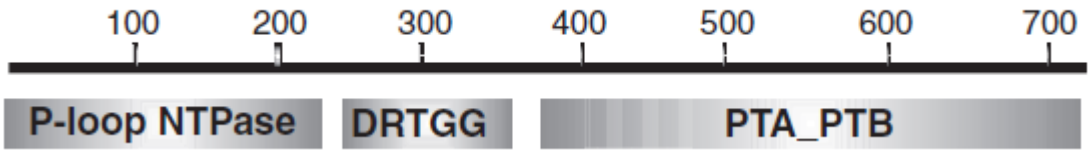

Figura 6 - Representação dos domínios conservados na enzima PTA de *E. coli*. Domínio P-*loop* NTPase, domínio DRTGG e domínio PTA_PTB. Fonte: Adaptado de CAMPOS-BERMUDEZ *et al*., 2010.

As moléculas de NADH, ATP, PEP e piruvato parecem ser reguladoras importantes de PTA tipo II de *E. coli*. Enquanto NADH e ATP inibem a atividade em ambas as direções, piruvato e PEP ativam formação de acetil fosfato e inibem a formação de acetil-CoA. *In vitro*, a enzima é 8 vezes mais ativa na direção de formação de acetil-CoA, porém as concentrações *in vivo* dos substratos e produtos de PTA quando *E. coli* cresce em glucose favorecem a síntese de acetil-fosfato (CAMPOS-BERMUDEZ *et al*., 2010).

1.5.1. Papel de PTA no "overflow metabolism"

O acetato é um dos produtos finais na produção de energia da maioria dos microrganismos anaeróbicos, geralmente é formado a partir de Acetil-CoA, o mecanismo de conversão depende do domínio ao qual o microrganismo pertence (SCHÄFER, SELIG, SCHÖNHEIT, 1993; SCHÖNHEIT e SCHÄFER, 1995). Em Bacteria, acetil-CoA é convertido a acetato por meio de duas enzimas: fosfotransacetilase (PTA; EC 2.3.1.8) e acetato quinase (Ack; EC 2.7.2.1) (BOCK *et al.,* 1999). PTA converte acetil-CoA e fosfato inorgânico a acetil-fosfato e CoA (acetil-CoA + fosfato $\rightleftharpoons$ CoA + acetil-fosfato) (ROSE *et al.,* 1954), e Ack converte acetil-fosfato e ADP em acetato e ATP (Figura 7) (ROSE *et al.,* 1954).

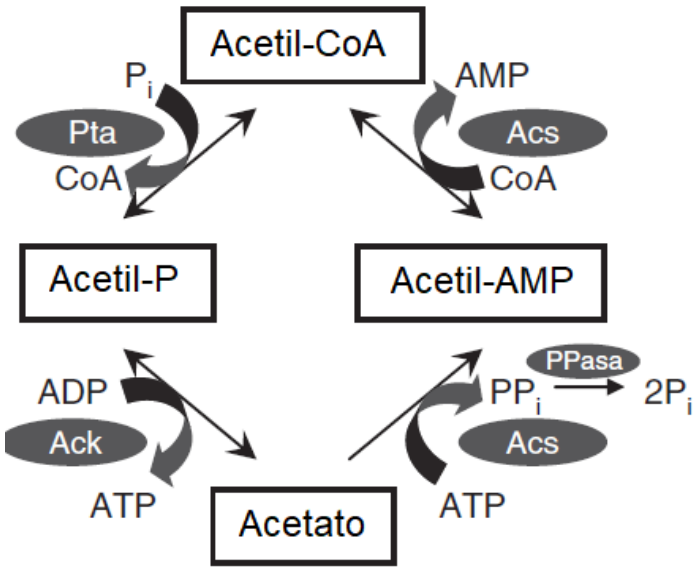

Figura 7 - Vias de produção de acetato em *E. coli*. As enzimas Ack (acetato quinase) e PTA catalisam uma via reversível de produção e assimilação de acetato. Fonte: Adaptado de CAMPOS-BERMUDEZ *et al*., 2010.

As reações PTA e Ack são muito importantes para o chamado "acetate switch", que viabiliza a transição entre a excreção e assimilação de acetato em um meio rico em nutrientes (WOLFE, 2005). Quando *E. coli* é cultivada em meio contendo glucose, esta bactéria converte parte da glucose em acetato, através do metabolismo fermentativo, utilizando uma via de menor eficiência de conversão energética mesmo na disponibilidade de oxigênio. Este efeito é conhecido como "Warburg effect" ou "Overflow metabolism" e é muito comum na natureza, ocorendo em células que se encontram sob rápida multiplicação, incluindo células cancerosas (HEINDEN, CANTLEY e THOMPSON, 2009). Quando a glucose no meio se esgota, *E. coli* aciona o "acetate switch" e inicia o processo reverso, utilizando agora acetato como fonte de energia, também com a participação das enzimas PTA e Ack (WOLFE, 2005).

Um enigma não resolvido na regulação metabólica é entender porque muitas células optam por realizar um metabolismo de menor eficiência de conversão de glucose em ATP quando estão sobre rápida proliferação. Várias hipótese têm sido levantadas como redução da capacidade respiratória por limitações físicas de espaço na membrana interna, necessidade de regeneração de NAD^+, limitação de CoA ou ainda uma maior eficiência na alocação de recursos energéticos para suprir necessidades de síntese protéica (YOU *et al.*, 2013).

Seja qual for a razão pela qual as células optam por metabolismo fermentativo na presença de glucose e oxigênio, os mecanismos que acionam este processo permanecem

elusivos para a ciência e são de fundamental importância para otimizar processos metabólicos e microorganismos e compreender o desenvolvimento de tumores.

1.5.2. Outras funções de PTA

Contiero *et al*. (2000) demonstraram que mutantes *pta* crescendo em meio rico em glucose exibem uma produção de acetato consideravelmente menor e por isso passam a excretar outros subprodutos normalmente não excretados, como piruvato, D-lactato e L-glutamato (CHANG *et al.*, 1999), isso ocorre pois em células crescendo em meio rico em glucose; piruvato e acetil-CoA são produzidos em excesso e não conseguem ser metabolizados devido à repressão de enzimas do ciclo TCA (CHANG *et al.,* 1999), assim a excreção de acetato é uma forma de equilíbrio e a via PTA/AcK possui papel importante nesta excreção.

Na reação catalisada por PTA é formado acetil-fosfato, em *E. coli,* acetil fosfato age como doador de fosforil para proteínas reguladoras de sistemas de dois componentes e parece ser um regulador global (AZAM *et al.,* 1999; BENNETT e HOLMS, 1975). Portanto, PTA parece ter um papel importante em várias vias do metabolismo bacteriano e o fato de MaeB de *A. brasilense* (AbMaeB) conter um domínio PTA pode significar seu envolvimento em vias similares.

Até o momento a maior parte dos estudos realizados com enzimas málicas tem como alvo organismos eucarióticos. Há alguns trabalhos de caracterização destas proteínas em procariotos, porém muito pouco ainda é esclarecido sobre elas nestes organismos. Os estudos com α proteobactérias são ainda mais escassos e não há nenhum trabalho caracterizando as enzimas málicas de *A. brasilense.* O mesmo ocorre com a enzima NAGK, apesar de sua interação com PII ser bem caracterizada em plantas e microrganismos fototróficos, ela ainda não foi estudada em *A. brasilense.*

A confirmação da relação de PII com ambas as proteínas poderia implicar em seu papel na produção de piruvato e NADPH, bem como na biossíntese da arginina, ampliando o papel conhecido desta proteína na regulação metabólica.

2. OBJETIVOS

Objetivo Geral

Caracterizar a interação entre a proteína PII (GlnZ) e os possíveis alvos selecionados: enzima málica $NADP^+$ dependente (MaeB) e N-acetil glutamato quinase (NAGK) de *A. brasilense*, a fim de compreender melhor o papel regulatório das proteínas PII.

Objetivos específicos

- ✓ Clonar os genes de *A. brasilense* que codificam as proteínas N-acetil glutamato quinase (NAGK) e a enzima málica $NADP^+$ dependente (MaeB1).
- ✓ Expressar e purificar as proteínas MaeB e NAGK;
- ✓ Testar a interação das proteínas alvos com PII em ensaios de coprecipitação *in vitro*;
- ✓ Caracterizar a enzima MaeB cineticamente;

3. CAPÍTULO I

In vitro characterization of the NADP$^+$ dependent malic MaeB enzyme and complex formation with GlnZ in *Azospirillum brasilense*

Introduction

The reversible oxidative decarboxylation of malate to pyruvate and CO_2 is catalyzed by the malic enzymes (ME). The ME reaction is associated with the reduction of NAD^+ to NADH (EC 1.1.1.38 and EC 1.1.1.39) or $NADP^+$ to NADPH (EC 1.1.1.40). ME are widely distributed in nature, they are present in organisms from all domains of life participating in key metabolic processes such as C4-photosynthesis carbon fixation in plants and lipid biosynthesis in mammals (Nelson and Cox, 2014). In prokaryotes, ME is part of the phosphoenol-pyruvate (PEP) / pyruvate / oxaloactetate node, which acts as a switch point for carbon distribution connecting glycolysis, gluconeogenesis and the TCA cycle reactions (Sauer and Eikmanns, 2005). Even though bacterial ME can catalyze both C3-carboxylation and C4-decarboxylation reactions, it is generally involved in the latter. Some prokaryotes can encode multiple ME with different catalytic and regulatory properties (Spaans *et al*., 2015). In *Escherichia coli,* two different ME were characterized namely, ScfA and MaeB. ScfA is a tetramer that can use both NAD^+ and $NADP^+$. On the other hand, MaeB forms a higher order oligomer (probably an octamer), is $NADP^+$ specific, and has an additional C-terminal non-catalytic phosphotransacetylase domain (PTA) (Bologna *et al.*, 2007). *Escherichia coli* MaeB ME activity is regulated by key metabolites and this modulation requires the C-terminal PTA domain which is also involved in MaeB oligomerization (Bologna *et al.,* 2007).

Two MaeB-like malic enzymes have been characterized in *Sinorhizobium meliloti*, a MaeB NAD^+ dependent (DME – diphosphopyridine nucleotide dependent) and a MaeB $NADP^+$ dependent (TME - triphosphopyridine nucleotide dependent) (Voegele *et al.*, 1999). Both are octamers containing a non-catalytic C-terminal PTA domain which is essential for MaeB oligomerization but not for the ME activity, suggesting that the non-catalytic PTA domain of MaeB may be involved in the metabolic control of MaeB. Both DME and TME MaeB isoforms are subject to regulation by a range of metabolites (Mitsch *et al.*, 1998; Voegele, 1993). Loss of these enzymes alters the levels of trehalose and putrescine in *Synorhizobium meliloti* (Zhang *et al.,* 2016) and leads to an accumulation of D-lactate in *Escherichia coli (*Zhang *et al.,* 2011). The DME but not the TME MaeB is essential for symbiotic nitrogen

fixation in *S. meliloti*. Only the ME domain of DME is essential for nitrogen fixation and the *E. coli sfcA* gene can complement nitrogen fixation of a *S. meliloti* DME mutant strain (Mitsch *et al.,* 2007).

The P_{II} family comprises a group of widely distributed signal transduction proteins found in nearly all Bacteria and also present in Archaea and in the chloroplasts of Algae and plants (Forchhammer, 2008). P_{II} forms barrel-like homotrimers with a flexible loop, namely T-loop, emerging from each subunit (Huergo *et al.*, 2013). P_{II} proteins have extraordinary sensory properties; they can exist in a number of structural status accordingly to the levels of ATP, ADP and 2-oxogluratate. These metabolites interact allosterically with P_{II} in three conserved binding sites located in the lateral cavity between each P_{II} subunit. ATP and ADP bind competitively to the nucleotide binding site, whereas the 2-oxoglutarate only interacts with P_{II} in the presence of Mg^{2+}ATP (Da Rocha *et al.*, 2013; Fokina *et al.,* 2010; Jiang and Ninfa, 2009; Truan *et al.,* 2010).

In Proteobacteria, P_{II} proteins are also subject to a cycle of reversible post translational modification (Merrick, 2014). Under a low nitrogen regime, the low intracellular glutamine level triggers the uridylyl-transferase activity of the bi-functional GlnD enzyme promoting the uridylylation of a conserved Tyr-51 located at the top of the P_{II} T-loop. Conversely, under a high nitrogen regime, accumulation of intracellular glutamine triggers the uridylyl-removing activity of GlnD and P_{II} proteins accumulate in its non-modified form (Araújo *et al.*, 2008; Jiang and Ninfa, 1999).

The ability of P_{II} to sense important metabolites and integrate signals deriving from energy status (ATP and ADP ratio), carbon (2-oxogluratate) and nitrogen (glutamine and 2-oxoglutarate) levels were capitalized during evolution such that P_{II} can act as a dissociable regulatory subunit of a range of transporters, transcriptional regulators and enzymes (Huergo, Chandra and Merrick, 2013; Huergo and Dixon, 2015; Forchhammer and Luddecke, 2016).

Recent P_{II} interactome analyses in the diazotrophic α Proteobacterium *Azospirillum brasilense* supports that P_{II} proteins may regulate a large number of protein targets, and MaeB was identified as a potential target of one of the P_{II} proteins encoded by this bacterium, namely GlnZ (Gerhardt *et al.,* unpublished).

In this work we present the biochemical characterization of MaeB from *A. brasilense* and *E. coli*. We show that both *E. coli* and *A. brasilense* have enzymatic active PTA domains and that L-glutamine is a potent activator of the malic enzyme activity of MaeB in both *E. coli* and *A. brasilense*. We show that MaeB forms a specific complex with the P_{II} protein GlnZ. The GlnZ-MaeB complex formation is regulated by the 2-oxoglutarate levels and by the GlnZ

uridylylation status. The ME activity of MaeB is activated in the presence of GlnZ. Our data suggest that P_{II} may act as a switch controlling the pace of carbon distribution between glycolysis, gluconeogenesis and the TCA cycle at the PEP/pyruvate/oxaloacetate node accordingly to the nutritional status of the cell.

Experimental Procedures

Bacterial strains, cloning and molecular biology methods

The bacterial strains used in this study are listed in Table 1. Isolation of plasmid DNA, gel electrophoresis, bacterial transformation and cloning were performed as described (Sambrook *et al*., 1989). Enzymes were obtained from commercial sources and used according to the manufacturers' instructions. DNA sequencing was performed using dye-labeled terminators in an automated DNA sequencer ABI 3500 from Applied Biosystems.

The *A. brasilense* FP2 MaeB1 gene was amplified by PCR using the *A.brasiliense* FP2 genomic DNA as template, the primers: 5' GAACCCATATGACCGAACCCGATACAAAGC 3' in combination with 5' CCGCCGGATCCTCACCAGGGCAGCGAGT 3' (Restriction sites for *Nde*I and *Bam*HI are underlined), and high-fidelity *Pfu*X7 DNA polymerase (Nørholm, 2010). The PCR products were digested with *Nde*I and *Bam*HI and ligated into pET29a or pET28a, previously digested with the same enzymes. The plasmids containing the AbMaeB1 gene were isolated and submitted to sequencing to confirm the identity of the gene. The plasmids were named pGAMaeB1 (pET29a expressing untagged MaeB1) and pGAMaeB1His (pET28a expressing N-terminal His tagged MaeB1).

The plasmid pMSA4ΔloopT expressing the version of GlnZ containing a deletion on the T-loop (GlnZΔ42-54) (Scarduelli, 2010) was used to generate a N-terminal His-tagged version of this enzyme, by subcloning *glnZ Nde*I and *Bam*HI fragment of pMSA4dellloopT into the *Nde*I and *Bam*HI sites of pET28a. The resulting plasmid was named pGAHisGlnZΔloop.

Protein methods

Electrophoresis of proteins was carried out by SDS-PAGE and gels were Coomassie blue stained. Protein concentrations were determined by the Bradford assay using bovine serum albumin as standard.

Sequence analysis

Protein sequences homologous and homeomorphic to *E. coli* MaeB (i.e. containing similar domain architecture, a malic domain fused to PTA domain) were retrieved from the PIRSF database (Nikolskaya *et al*., 2006). Sequences were aligned using Clustal W (Thompson *et al.,* 1994), gaps were removed from the alignment and a Neighbor Joining phylogenic tree was generated using MEGA 7 (Kumar *et al.*, 2011) and 1,000 bootstraps.

Expression and purification of proteins

Untagged MaeB1 from Azospirillum brasilense

E. coli BL21 (λDE3) carrying the pGAMaeB plasmid was cultivated in LB (37ºC at 200 rpm) containing kanamycin (50 $\mu g.ml^{-1}$) to an OD of 0.6. Protein expression was induced by adding 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were kept at 20ºC, 200 rpm for 20 hours. Cells were harvest by centrifugation, ressuspended in Buffer 1 (50 mM Tris HCl pH 8; 100 mM NaCl), disrupted by sonication and centrifuged (20.000xg, 15 min at 4ºC). The soluble fraction was loaded onto a 5 ml Heparin column (GE-healthcare) which was pre-equilibrated with Buffer 1. Bound proteins were eluted with a NaCl gradient (100 to 1,000 mM of NaCl in buffer 1). Fractions containing AbMaeB1 were pooled and the buffer was exchanged using a 20 ml desalting column. Proteins were stored in 50 mM Tris-HCl pH 8; 100 mM NaCl; 10% glycerol and 0.1 mM DTT at -40ºC.

His tagged MaeB, truncated his tagged PTA from Escherichia coli and his tagged MaeB from Azospirillum brasilense

E. coli BL21 (λDE3) carrying the pET-MaeB, pET-MaeBPTA and pGAMaeBHis plasmid were cultivated in LB (37ºC at 200 rpm) containing kanamycin (50 $\mu g.ml^{-1}$) to an O.D of 0.6. Protein expression was induced by adding 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were kept at 20ºC, 200 rpm for 20 hours.

Cells were harvested by centrifugation for 5 min at 2240xg; ressuspended in buffer 1 (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 50 mM imidazole). The cells were disrupted by sonication and centrifuged for 10 min at 20000xg at 4°C. The soluble fraction was loaded onto a 5 ml Hitrap chelating Ni^{2+} column (GE Healthcare) pre-equilibrated in buffer 1. The elution was carried in buffer 1 using an imidazole gradient (50 to 1000 mM imidazol). Fractions

containing the proteins of interest were pooled and buffer was exchanged to 50 mM Tris-HCl pH 7.5, 10% glycerol, 100 mM NaCl using a 5 ml desalting column (GE Healthcare). The proteins were stored in small aliquots at -40ºC.

Purification of GlnZ

The expression and purification of His-GlnZ was carried out as described in Moure *et al.* (2012). His-GlnZΔ42-54 was purified using the same protocol. The purification of untagged GlnZ was performed as described in Huergo *et al.* (2007).

Purification of GlnD

The GlnD protein from *E. coli* was purified according Rodrigues (2017) and kindly provided by Dr. Thiago E. Rodrigues.

In vitro uridylylation of His-GlnZ

Purified His-GlnZ was subjected to *in vitro* uridylylation in a 500 µl reaction mixtures containing 100 µM His-GlnZ (considered as monomer), 1 mM ATP, 2 mM UTP, 5 mM 2-ketoglutarate, 1 µM of purified *E. coli* GlnD. The reaction was performed using 100 mM Tris–HCl pH 7.5, 100 mM KCl and 25 mM $MgCl_2$ as buffer. The reactions were incubated at 37ºC overnight and terminated by heating at 60ºC for 15 min to allow GlnD denaturation which as further removed by centrifugation. The His-GlnZ-UMP_3 was dialyzed against buffer (50 mM Tris HCl pH 8.0, 100 mM KCl, glycerol 10%) and stored in small aliquots at -40ºC. The full uridylylation His-GlnZ was determined by Native-PAGE and by MALDI-TOF mass spectrometry as described previously (Inaba, 2009).

Gel filtration chromatography

Analytical gel filtration chromatography was performed on a Superose 6 10/30 column (GE Healthcare) using 50 mM Tris-HCl pH 8,0 100 mM NaCl and 5% glycerol (v/v) as buffer. The column was calibrated with the following molecular mass markers from Bio-Rad: Thyroglobulin (670 kDa); gama-globulin (158 kDa); ovalbulmin (44 kDa) and myoglobin (17 kDa). The runs of the purified proteins were performed at room temperature at a flow rate of 0.5 ml per minute.

Protein co-purification using magnetic beads

In vitro complex formation was performed using Ni^{2+} Magnetic beads (Promega) as described (Huergo *et al.*, 2007). All reactions were conducted in 50 mM Tris-HCl pH 8, 100 mM NaCl, 10% glycerol, 20 mM imidazole in the presence or absence of effectors and a non-denaturing detergent (10% Nonidet) as indicated in each experiment. Four µl of beads were equilibrated by three washes with 200 µl of buffer. Binding reactions were performed in 400 µl of buffer by adding purified proteins (20 µg of tagged protein and 50 µg of the untagged protein). After 15 minutes, the beads were washed 3 times with 200 µl of buffer. Then, the samples were mixed with sample buffer and analyzed in 15% SDS-PAGE stained with Coomassie blue.

In vitro MaeB malic activity

Spectrophotometric assay

The reactions were performed using a microplate spectrophotometer (Biotek® Epoch) at 340 nm to detect the formation or consumption of NADH or NADPH. Reactions were followed by 10 min and initial velocities were calculated from the linear range of each assay.

The oxidative decarboxylation of L-malate by MaeB was measured in the following buffer: 50 mM Tris HCl pH 7.5, 0.5 mM $NADP^+$, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA, 40 mM of L-malate and 0.1 µM MaeB (considering the monomer mass). The reactions were started by addition of L-malate. NAD^+ substituted $NADP^+$ when indicated. The Michaelis-Menten constant of L-malate was determined by varying its concentration, while other components remain at unchanged concentrations (50 mM Tris HCl pH 7.5, 0.5 mM $NADP^+$, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA and 0.1 µM MaeB). The kinetic parameters were calculated at least in duplicate using GraphPad Prism 7.0 program.

The direct malic reaction of MaeB was evaluated using $NADP^+$ 0.5 mM and L-malate 40 mM, in the presence of different metabolites at concentrations near the physiological range described for *E. coli* (Bennett *et al.*, 2009); Acetyl-P 1 mM, Acetyl-CoA 0.6 mM, Coenzyme A 1.5 mM, Aspartate 4.5 mM, Glutamate 100 mM, ADP 1 mM, ATP 1 mM, 2OG 1.5 mM, Glutamine 1.5 mM. The effect of KCl, $MgCl_2$ and $MnCl_2$ was also determined. The results were showed as relative activity compare to the regular reaction in the absence of effectors as 1 (standard reaction).

The ability of MaeB to catalyze the reverse reaction (carboxylation of pyruvate) was assayed in: 50 mM Tris-HCl pH 7; 30 mM $NaHCO_3$, 30 mM pyruvate, 10 mM $MgCl_2$, 0.1 mM NADPH and 0.1 µM MaeB. The reactions were started by the addition of pyruvate.

LC-MS assays

LC-MS assays were performed in order to identify the substrates and products of malic and PTA activities. The direct malic reaction mix was composed by: 50 mM Tris-HCl pH 7.5, 0.5 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM NaCl, 0.1 mM dithiothreitol (DTT) and 0.1 µM AbMaeB1. The reverse malic reaction mix was composed by: 50 mM Tris-HCl pH 7, 30 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.1 mM NADPH, 0.1 mM DTT, 0.1 µM AbMaeB1. The PTA reaction mix was composed by: 20 mM Tris-HCl pH 8; 1 mM coenzyme A, 5 mM $MgCl_2$, 0.1 mM DTT and 0.1 µM MaeB. Reactions were started with the addition of L-malate 40 mM, pyruvate 30 mM or acetyl phosphate 10 mM (direct malic reaction, reverse malic reaction and PTA reaction, respectively) and incubated at 30ºC. Samples of 80 µl were removed after 0, 5, 15 and 30 minutes and mixed with 20 µl of ice-cold acetic acid 10 % (v/v) to stop the reactions. Samples were centrifuged at 20.000xg, 5 min at 4ºC and 10 µl of supernatant was used for LC-MS analysis as described (Rodrigues *et al*., 2014). The formation or utilization of NADPH or the formation of Acetyl-CoA (depending on the reaction mix) were analyzed by extracting their calculate mass in negative mode (M-H) using an error window of 0.01 m/z.

Results

Sequence analysis of A. brasilense MaeB1

In our previous P_{II} interactome studies we have identified a protein annotated as malic enzyme MaeB (GI-392380750) as a potential binding target of the P_{II} protein GlnZ in *A. brasilense*. When we compared the sequence of *E. coli* MaeB against the genome of *A. brasilense* Sp245 using tblastn (Altschul *et al.,* 1997) we noted that *A. brasilense* encodes two proteins with high similarity with *E. coli* MaeB. The one identified in our previous interactome study was named MaeB1 (AbMaeB1); it is encoded by the main chromosome and shares 47% identity with *E. coli* MaeB. The other is encoded by the plasmid p1, is 58% identical to *E. coli* MaeB and was named MaeB2 (AbMaeB2). AbMaeB1 and AbMaeB2 are 47% identical (Fig S1). Both MaeB1 and MaeB2 have a domain organization similar to *E. coli* MaeB, a N-terminal malic enzyme domain followed by a C-terminal PTA domain (Fig. S1).

Analysis of *E. coli* MaeB homologous and homeomorphic (containing similar domain architecture) using the PIRSF database identified 8180 sequences with homologues with similar architecture (PIRSF code 036684). All sequences belong to Bacteria and Archaea, with the exception of one sequence derived from Fungi. Phylogenetic analysis revealed that AbMaeB1 clustered with *S. meliloti* TME while AbMaeB2 clustered with *S. meliloti* DME. *E. coli* MaeB was more distant related clustering along with sequences from other Enterobacteria (Fig. S2).

Characterization of AbMaeB1

The gene encoding *A. brasilense* MaeB1 was cloned into pET29a, the protein was expressed in *E. coli* and purified to homogeneity on SDS-PAGE (Fig. 1A). Gel filtration chromatography showed that MaeB1 elutes as a symmetric peak at a volume corresponding to a molecular weight of 710 kDa (Fig. 1B and C). The predicted molecular weight of the MaeB1 polypeptide is 83 kDa, hence, MaeB1 is also arranged in a high molecular weigh oligomer as observed in *E. coli* and *R. meliloti*, probably forming an octamer.

Initial assays were performed to detect the *in vitro* activity of MaeB1. Purified MaeB1 was mixed with 40 mM L-malate in the presence of either 0.5 mM of $NADP^+$ or NAD^+. The ME activity was determined by measuring the formation of NADPH or NADH at 340 nm. In the presence of $NADP^+$, a rapid formation of NADPH (nmol/s) was observed while a minor increase in the formation of NADH (nmol/s) was detected using NAD^+ as electron acceptor (Fig. 2). These data suggest that MaeB1 preferentially uses $NADP^+$ as cofactor. Indeed, sequence analysis (Fig. S1) showed a conserved motif characteristic of $NADP^+$ dependent ME in *A. brasilense* MaeB1 (Bologna *et al*., 2007) and AbMaeB1 clustered with TME MaeB from *S. meliloti* (Fig. S2).

Attempts were made to determine the reverse malic reaction using 30 mM of pyruvate, 30 mM $NaHCO_3$ and 0.1 mM of NADPH or NADH as substrates. Reactions were incubated at 30ºC, but no significant changes in absorbance were detected (not shown). Hence, AbMaeB1 cannot convert pyruvate to malate under the assay conditions similar to TME MaeB of *S. meliloti* (Voegele *et al.,* 1999).

Kinetic analyses were performed with AbMaeB1 by plotting the initial velocities against increasing concentrations of L-malate. A typical Michaelis-Menten hyperbolic response was observed (Fig. 3). The K_M for malate is 8.55 ± 1.34 mM, Vmax of the reaction is 5.5 ± 0.33 nmol/s, and the k_{cat} value is 183.4 ± 11.16 s^{-1}. The K_M for malate of AbMaeB1 is in the same range reported for other organisms (3.4 mM for *E. coli* MaeB (Bologna *et al.,* 2007) 2.6 mM for *Rhizobium subtilis* (Voegele *et al.,* 1999); 3.8 mM for *Corynebacterium glutamicum*

(Gourdon *et al.,* 2000). The K_M for malate of AbMaeB1 is also within the range of intracellular malate concentrations measured in *E. coli* under different carbon regimes (1.7 to 3.5 mM) (Bennett *et al.,* 2009).

Effect of different metals and metabolites on AbMaeB1 malic activity

Previous studies indicated that Mg^{2+} is required for *E. coli* MaeB activity but enzyme inhibition was detected in Mg^{2+} concentrations above 4 mM (Bologna *et al.,* 2007). We tested different concentrations of $MgCl_2$ and $MnCl_2$ under saturating L-malate (40 mM). The results indicate that Mg^{2+} and Mn^{2+} are essential to the malic activity of AbMaeb1, with a preference for Mn^{2+} (Fig. 4), this is in agreement with the results obtained with EcMaeB (Brown and Cook, 1981). We also noted that when the concentration of Mn^{2+} and Mg^{2+} were increased, the AbMaeB1 activity was proportionally smaller (Fig. 4), the same response was observed for Mg^{2+} in EcMaeB (Bologna *et al.*, 2007). Given the estimated intracellular concentration of Mg^{2+} in bacteria of 30 mM which is 100 fold higher than Mn^{2+} (0.3 mM) (Gotto, 1982) we opted to use Mg^{2+} 5 mM in all subsequent experiments.

The MaeB from *E. coli* and *S. meliloti* are subject to regulation by several metabolites (Bologna *et al.*, 2007; Voegele *et al.*, 1999). Hence, we tested the effects of different metabolites at physiological relevant concentrations on the malic activity of AbMaeB1. Strong activation was observed with KCl and glutamine, increasing the malic activity up to 6 and 8-fold, respectively (Fig. 5). Strong inhibition was detected in the presence of acetyl-P and coenzyme A and slight inhibition with ATP combined with 2-OG (Fig. 5).

Activation by KCl was previous reported for EcMaeB (Bologna *et al.*, 2007). Aspartate activated EcMaeB but no effect was observed with AbMaeB1 (Fig. 5). Acetyl-P activated EcMaeB (Bologna, Drincovich and Andreo, 2007) while AbMaeB1 is strong inhibited by this compound (Fig. 5). Inhibition by CoA was observed in nearly all MaeB-like enzymes previously characterized with the exception of *S. meliloti* DME MaeB (Bologna *et al.*, 2007; Voegele *et al.,* 1999).

Given the strong activation of AbMaeB1 by L-glutamine and the central role of this metabolite in nitrogen signaling (van Heeswijk *et al.,* 2013) we further investigated the effect of L-glutamine on both AbMaeB1 and EcMaeB. We noted that the activation of AbMaeB1 by glutamine was dose dependent (Fig 6A) providing a typical hyperbolic Michaelis-Menten response with K_{act} of 1.98 mM L-glutamine. Interestingly, L-glutamine was also able to active EcMaeB enzyme (Fig 6B) with a K_{act} of 0.74 mM. The determined values of L-glutamine K_{act}

for both enzymes fit well within the reported physiological range of this metabolite reported in both *E. coli* and *A. brasilense.*

The PTA domain of both EcMaeB and AbMaeB1 is catalytic active

Previous studies with EcMaeB and *S. meliloti* DME and TME MaeB failed to detect the activity of the PTA domain in these proteins. We analyzed the functionality of the PTA domain of AbMaeB1 by LC-MS. Surprisingly; we noted that AbMaeB1 was able to produce Acetyl-CoA when incubated with acetyl-P and CoA (Fig. 7). The same PTA activity was observed when using EcMaeB (Fig. 7). This is in stark contrast with the results obtained by Bologna, Andreo and Drincovich (2007). In the previous work, the EcMaeB PTA activity was determined using a spectrophotometric assay and an engineered N-terminal His tagged form of EcMaeB. We used the very same His tagged version of EcMaeB used by Bologna, Andreo and Drincovich (2007) and we noticed that the presence of the N-Terminal His tagged on EcMaeB reduce the PTA activity significantly (Fig. 7). After 30 min of incubation, the intensity of the Acetyl-CoA peak was 5 x 10^4 and 0.3 x 10^4 for untagged EcMaeB and His tagged EcMaeB, respectively (Fig. 7). The reduced activity of the His Tagged EcMaeB together with the use of a less sensitive spectrophotometric assay may explain why EcMaeB PTA activity was not detect in the previous study.

Characterization of the A. brasilense MaeB1-GlnZ complex

In order to characterize complex formation between GlnZ and MaeB, co-purification assays were performed using Ni^{2+} beads and His-GlnZ as a bait and untagged MaeB1 as target. Control assays indicated that untagged MaeB1 was not retained by the Ni^{2+} beads (Fig. 8, lanes 1, 3, 5 and 7). However, when His-GlnZ was present in the assay, a strong signal of MaeB1 was observed in the elution of the Ni^{2+} beads co-purification assay (Fig. 8, lanes 2, 4 and 6), these data indicate that MaeB1 is able to form a complex with His-GlnZ.

The signal of MaeB1 in the elution was stronger when the co-purification assay was performed in the presence of 1 mM ADP or 1 mM ATP (Fig. 8, lanes 4 and 6). When 1 mM ATP and 1.5 mM 2-OG were combined, MaeB1 did not co-purify with His-GlnZ (Fig. 8, lane 8). These data indicate that complex formation between MaeB1 and GlnZ is stimulated by nucleotides and that the presence of 2-OG inhibits complex formation. The negative effect of 2-OG in MaeB1-GlnZ complex was confirmed by the partial dissociation of MaeB1 from the His-GlnZ coated Ni^{2+} beads when 1 mM ATP was combined with 1.5 mM of 2-OG (Fig. S3). This effect was not observed when 1 mM ADP was combined with 1.5 mM of 2-OG (Fig. S3).

Given that GlnZ can only bind 2-OG in the presence of MgATP but not in the presence of MgADP (Truan *et al*., 2010;Truan *et al.,* 2014) we conclude that the binding of 2-OG changes the GlnZ structure in a way that favors its dissociation of MaeB1. The same assay was performed with His-AbGlnB and AbMaeB1, but none complex formation could be detected, which may mean that this interaction is specific of GlnZ despite the high similarity between GlnZ and GlnB.

The complex PII-MaeB seems not to be conserved in E. coli

We evaluated if the complex formation between MaeB and PII proteins would be also conserved in *E. coli*. To this end, we used His-tagged *E. coli* GlnB or GlnK as bait to test the interaction against either EcMaeB or AbMaeB1. No interaction could be detected, suggesting that *E. coli* MaeB is not able to bind PII proteins (data not shown).

The UMP group affects the complex formation

To evaluated if the uridylylation of the GlnZ would affect complex formation with MaeB1, His-GlnZ was subjected to *in vitro* uridylylation and purified by desalting to remove excess of UTP, ATP and 2-OG present in the uridylylation reaction. The modified GlnZ was in its fully uridylylated form (GlnZ-UMP_3) as judged by MALDI-TOF mass spectrometry analysis (Fig. 9). When GlnZ-UMP_3 was mobilized in the Ni^{2+} beads, MaeB1 could not be co-purified in any of the conditions tested (Fig 10). These data support that the presence of UMP group at the GlnZ T-loop affect the ability of GlnZ to interact with MaeB1.

Role of the GlnZ T-loop in the GlnZ-MaeB1 complex formation

Since the GlnZ-UMP_3 was not able to interact with MaeB1, we thought that the T-loop would be important for complex formation. To address this hypothesis, we performed the co-purification assays using a His-GlnZ with a partial deletion in the T-loop. The results depicted in Figure 11 indicate that His-GlnZΔloop interacted with MaeB1 in all the conditions tested. Hence, the GlnZ T-loop is not essential for the complex formation. Interestingly, the interaction between His-GlnZΔTloop and AbMaeB1 also occurred when ATP and 2-OG were combined (Fig. 11), we speculate that the conformation adopted by the T-loop when ATP and 2-OG acts as a switch to dissociate the MaeB1-GlnZ complex, and such switch would be absence in a His-GlnZΔTloop variant. Alternatively, the His-GlnZΔTloop may have reduced affinity for 2-OG binding.

The malic activity of AbMaeB1 seems to be controlled by GlnZ protein

The direct malic reaction of AbMaeB1 was analyzed by measuring the formation of NADPH by LC/MS in the presence or absence of excess GlnZ (Fig 12). The results showed that after 15 min incubation, the amount of NADPH formed is higher in the reaction with GlnZ than without it (Fig. 12), this result is confirmed by the amount of $NADP^+$ consumed in the same reactions (Fig. 12). These preliminary data suggests that the interaction between GlnZ and AbMaeB1 leads to the activation of the ME.

Discussion

In this study we characterized the enzyme AbMaeB1 from *A. brasilense*. Gel filtration analysis suggests that this enzyme forms a high order oligomer, probably an octamer (Fig. 1), which is in agreement with the data reported for other MaeB-like enzymes (Bologna *et al*., 2007). Regulatory enzyme are usually arranged in complex oligomeric forms, in fact we noted that the ME activity of AbMaeB1 is subject to complex regulation by small metabolites as also observed in the *E. coli* orthologue (Bologna *et al.,* 2007)

The AbMaeB1 ME activity is subjected to a strong activation by L-glutamine (Fig 5) and this same effect was detected in EcMaeB (Fig. 6 B), in both cases the determined activation constants fit well within the reported physiological range of glutamine determined in *E. coli* and *A. brasilense* supporting the physiological significance of glutamine regulation. The phylogenetic tree of the MaeB-like enzymes shows that EcMaeB and AbMaeB1 are well separated into different subgroups (Fig. S2). Hence, the conservation of the response to glutamine in EcMaeB and AbMaeB1 suggests that glutamine modulation evolved within a common ancestor of the MaeB-like enzymes and it is tempting to speculate that such modulation is likely to occur in other MaeB-like enzymes. Still, the activation by NH_4^+ was already observed in some organisms (Gourdon *et al.,* 2000; Lamed and Zeikus, 1981; Garrido-Petierra *et al.,* 1983; Kawai *et al.,* 1996), and since glutamine is a sensor of NH_4^+ levels, we can suppose that the enzymes of these organisms could also be regulated by this metabolite.

It is well established that the intracellular glutamine levels in Proteobacteria correlates with nitrogen availability and this molecule acts as a major signal to regulate the expression of genes and activity of central enzymes in nitrogen metabolism (Dixon *et al*., 2004; Huergo *et al.,* 2013; Huergo and Dixon, 2015; van Heeswijk *et al.,* 2013). In *E. coli* and *A. brasilense,* ammonium limitation results in reduced glutamine pool. Conversely, a rapid glutamine increase is observed upon an ammonium shock within seconds (Huergo *et al.,* 2009).

The major operating ammonium assimilation route in both *E. coli* and *A. brasilense* is GS-GOGAT (Fig S4). After glutamine production by GS, GOGAT requires reducing power in the form of NADPH to convert glutamine into glutamate which, in turn, acts as a major nitrogen donor for other biosynthetic reactions (van Heeswijk *et al.,* 2013). The activation of MaeB by glutamine could help to provide the required NADPH for ammonium assimilation (Fig. S4). In addition, MaeB could provide reducing power for other key biosynthetic reactions, for instance the high NADPH demanding fatty acid biosynthesis (Liang and Jiang, 2015; Rodriguez *et al.,* 2012).

Our findings shows that MaeB has the ability to sense the levels of both carbon (in the form of L-malate accordingly to the enzyme K_M) and nitrogen (in the form of L-glutamine accordingly to the enzyme K_{act}). Hence, as long as carbon and nitrogen building blocks are present, MaeB ME will operate at maximum speed providing NADPH to fuel biosynthetic reactions.

The activation of MaeB ME by glutamine will impact not only the NADPH / $NADP^+$ ratio but also in carbon distributions between TCA / glycolysis / gluconeogenesis (Fig S4). *A. brasilense* is typically found associated with plants roots and preferentially uses organic acids such as malate as carbon sources (Day and Döbereiner 1976; Okon, Albrecht, and Burris 1976). In fact, L-malate is one of the major carbon compounds excreted by plants in the rhizosphere (Driscoll and Finan, 1993). The activation of MaeB ME by glutamine may help to trigger gluconeogenesis (converting L-malate into the gluconeogenesis precursor pyruvate) and thereby produce carbohydrates building blocks needed for cell replication only if nitrogen is available.

We attempted to measure the reverse malic reaction activity of AbMaeB1 using LC/MS, the preliminary data suggest that this activity is very slow (not shown) which is in agreement with previous studies using EcMaeB were the reverse reaction is apparently much less significant that the forward reaction (Bologna *et al*., 2007).

Carbon distribution through MaeB has another level of complexity if we consider the functionality of the PTA domain identified for the first time in this work. We demonstrated that both AbMaeB and EcMaeB can produce Acetyl-CoA when incubated in the presence of CoA and Acetyl-P. At the time we still do not know whether the enzyme also catalyzes the reverse reaction and if glutamine affects the MaeB PTA activity.

The concerted action of MaeB ME and PTA activities along with pyruvate dehydrogenase and pyruvate kinase would allow conversion of malate into acetate producing ATP (and NADPH) (Fig S4) and without the need of the costly synthesis of all proteins

necessary for the operation of the respiratory chain. This pathway would allow the utilization of abundant malate by *A. brasilense* in the rizosphere to produce ATP without the need of oxygen, such metabolic strategy could be useful when biological nitrogen fixation is operating given the high ATP demand and oxygen sensitivity of nitrogenase. Only the full characterization of the PTA domain of MaeB along with *in vivo* analysis will allow a better interpretation of MaeB function on carbon distribution and/or energy production.

In addition to the already complex nature of MaeB, Gerhardt *et al*. (unpublished) identified AbMaeB1 as a potential new binding target of one of the P_{II} proteins encoded by *A. brasilense,* namely GlnZ. Here, we characterized a complex formation between the GlnZ and AbMaeB1. We demonstrated that the complex formation is regulated by the 2-oxoglutarate levels and by the GlnZ uridylylation status. Since the interaction is destabilized by the addition of 2-OG and occurs only when GlnZ is deuridylylated, we conclude that GlnZ binds to AbMaeB1 in conditions of high nitrogen (high intracellular glutamine). Given that GlnZ is poorly expressed under high nitrogen (De Zamaroczy, 1998) we speculate that GlnZ may have a role in the regulation of MaeB1 upon ammonium shock as occurs with AmtB and DraG (Huergo *et al.*, 2006a, 2006b, 2007, 2009; Rajendran *et al.,* 2011).

Our preliminary data suggests that AbMaeB1 ME is activated upon interaction with GlnZ. Hence, GlnZ would promote AbMaeB1 ME activity upon an ammonium shock. At the time, we still do not know how the activation of AbMaeB1 ME by glutamine correlates with the activation by GlnZ. In case these two effects are additive, one would expect a very high AbMaeB1 ME activity after an ammonium shock and this could facilitate ammonium assimilation by providing NADPH as discussed earlier. The concerted action of modulation by glutamine and GlnZ could allow fine tuning of MaeB activity in response to glutamine, 2-OG and ATP/ADP ratio. Based on these observations, we propose a regulation model to address the importance of GlnZ and glutamine in MaeB activity (Fig. S5).

From a structural point of view, the AbMaeB1-GlnZ does not require the GlnZ T-loop; indeed a GlnZΔT*loop* variant was able to form a complex with AbMaeB1 in all effector conditions. We conclude that the T-loop is not essential for the interaction and the binding may occur in another region of GlnZ. The fact of the interaction between AbMaeB1 and GlnZΔT*loop* occurs even in the presence of 2-OG suggests that in the wtGlnZ the binding of 2-OG results in a T-loop conformation that negatively affect the docking site of AbMaeB1.

We were not able to detect complex formation between AbGlnB/AbMaeB1, EcGlnK/EcMaeB, EcGlnB/EcMaeB and EcMaeB/AbMaeB1. These data suggest that the

interaction is only conserved in *A. brasilense* but not in *E. coli* and complex formation is specific to GlnZ and not GlnB.

In summary, the identification of the forward PTA activity of MaeB indicates that this multifunctional enzyme may plays much broader role in metabolism acting not only as a key component of the PEP / pyruvate / oxaloacetate node and NADPH / $NADP^+$ ratio but also playing a pivotal role in acetate metabolism. Regulation of MaeB ME activity by glutamine is apparently a conserved property of this class of enzyme and this finding provides a novel link between nitrogen and carbon metabolism. Our data suggest that PII protein GlnZ could be modulating MaeB1 ME activity in *A. brasilense* by direct protein interaction and this may help to provide NADPH required for ammonium assimilation.

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Tables and Figures

Table 1 – Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Source/reference
E. coli		
BL21 (λDE3)	hsdS, gal(λclts 857 ind1 Sam7 nin5 lacUV5-T7 gene 1)	Sambrook *et al.*, 2001
DH10B	Sm^r ; F' [*pro*AB^+ *lacZ*M15]	Invitrogen
A. brasiliense		
FP2	Nal^rSm^r Wild strain	
SP7	Nif+	Pedrosa e Yates , 1984
Plasmíds		
pET28a	Km^r. Expression vector T7 promoter	Novagen
pET29a	Km^r. Expression vector T7 promoter	Novagen
pMSA4	Km^r (pET29a). Expresses the *A. brasilense* GlnZ	Moure *et al.,* 2012
pMSA4 dellloopT	Km^r . Expresses the *A. brasilense* GlnZ with deletion of T loop (Δ42-54). T7 promoter.	Scarduelli, 2010.
pGAHisGlnZΔ*loop*	Km^r (pET28a). Expresses the A. brasilense GlnZΔ*loop* carrying a 6x His tag at N-terminal	This work
pMSA3	Km^r (pET28a). Expresses the *A. brasilense* GlnZ carrying a 6x His tag at N-terminal	Araujo *et al.*, 2004)
pET-MaeB	Amp^r Expresses the *Escherichia coli* MaeB carrying a 6x His tag at C-terminal	Bologna *et al.,* 2007
pET-MaeBPTA	Amp^r Expresses the *Escherichia coli* truncated PTA carrying a 6x His tag at C-terminal	Bologna *et al.,* 2007
PGAMaeB	Km^r (pET29a) Expresses the *Azospirillum brasilense* MaeB	This work
pGAMaeBHis	Km^r (pET28a) Expresses the *Azospirillum brasilense* MaeB carrying a 6x His tag at N-terminal	This work

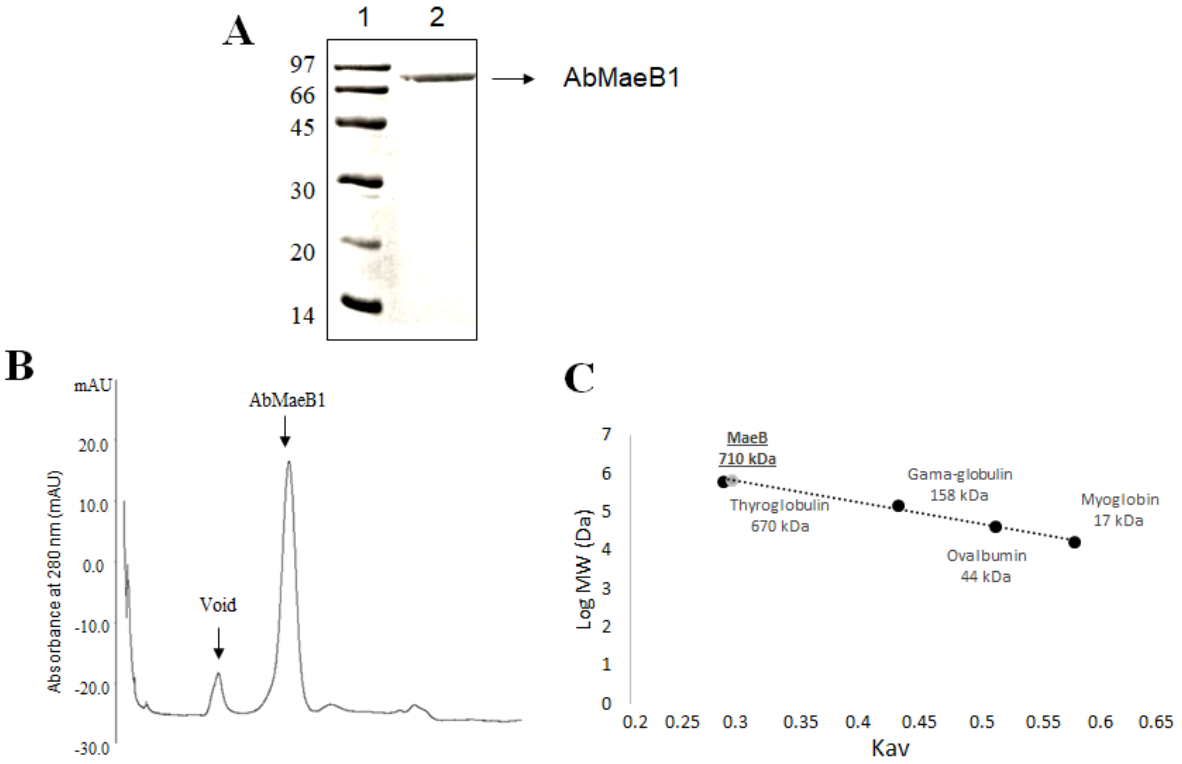

Figure 1 - Analysis of purified *A. brasilense* MaeB1. A) SDS-PAGE analysis of AbMaeB1: Lane 1 - MW Standards in kDa. Lane 2 - 1µg of the purified AbMaeB1. The gel was Coomassie Blue stained. B) Elution profile of purified AbMaeB1 on a Superose 12 10/30 GL column. C) Log MW vs Kav plots of molecular mass standards (BioRad) and AbMaeB1.

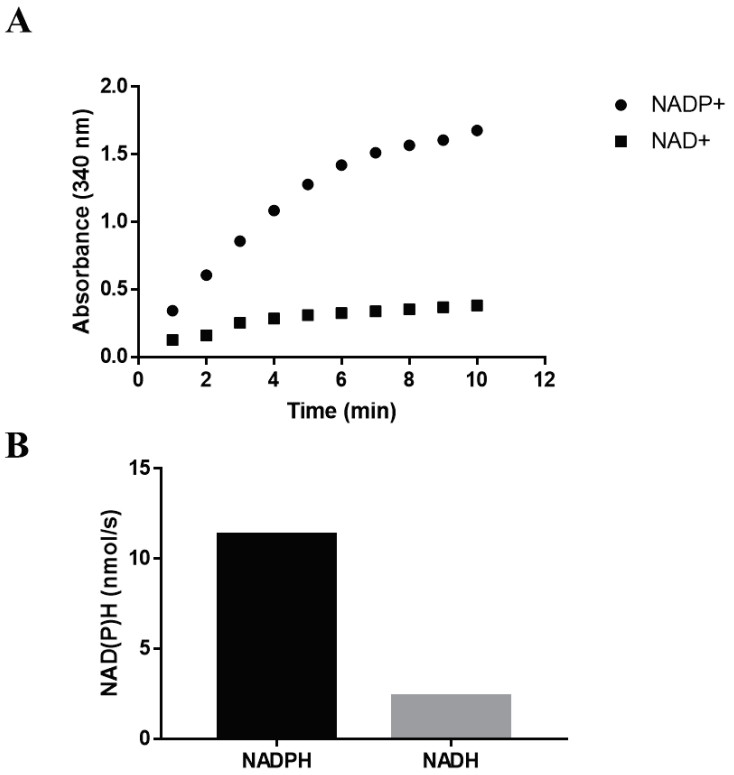

Figure 2 - AbMaeB1 activity in the presence of $NADP^+$ or NAD^+. (A) The activity of AbMaeB1 was determined by measuring the increase in the absorbance (340 nm) and (B) analyzing the formation of NADPH or $NADP^+$ (nmol/s). The linear range of the data in A was used to determine the initial velocities of MaeB1 activity in the presence of 40 mM of L-malate and 0.5 mM of NAD^+ or $NADP^+$ as indicated.

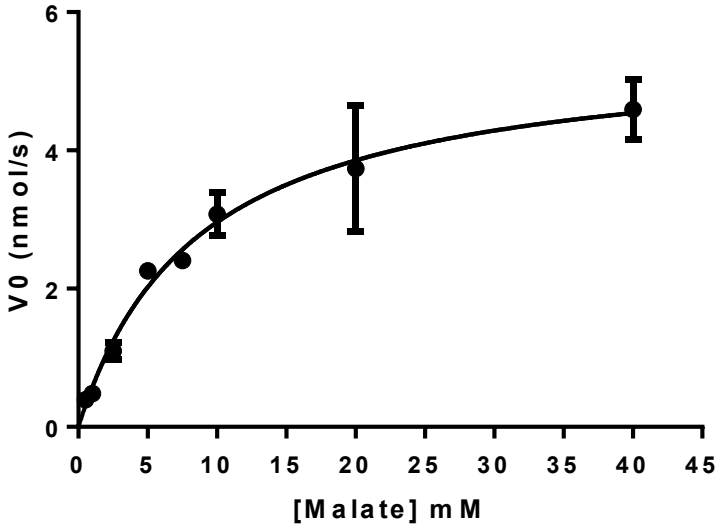

Figura 3 - Determination of kinetic parameters for AbMaeB1. Initial velocity (V_0) vs L-malate concentration. Average data from triplicate experiments ± SD.

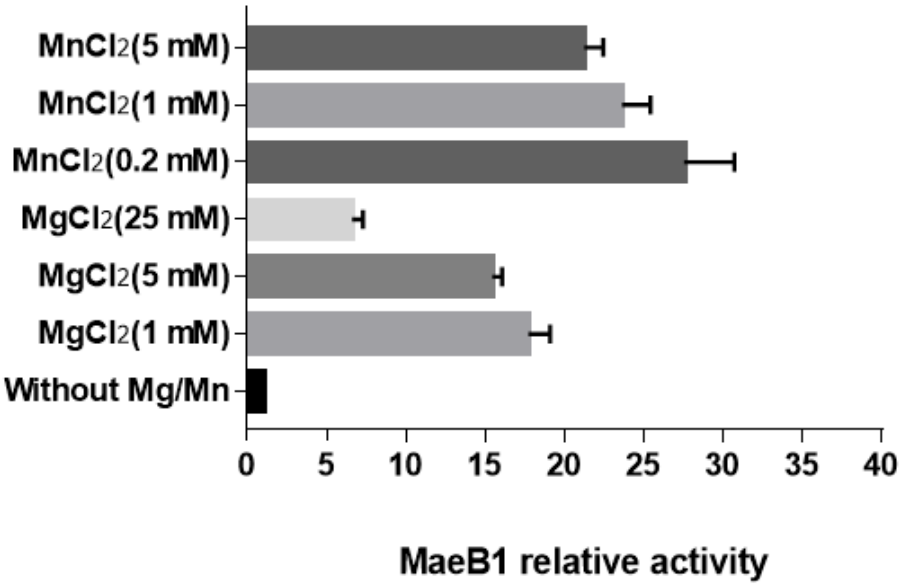

Figure 4 – Effect of Mg^{2+} and Mn^{2+} on AbMaeB1 malic enzyme activity. ME activity was measured in the presence of different concentrations of $MgCl_2$ and $MnCl_2$ as indicated and 40 mM of L-malate and 0.5 mM of $NADP^+$. Results were plotted as % of activity in the presence of $MgCl_2$ 1 mM (100%). Average data from triplicate assays ± SD.

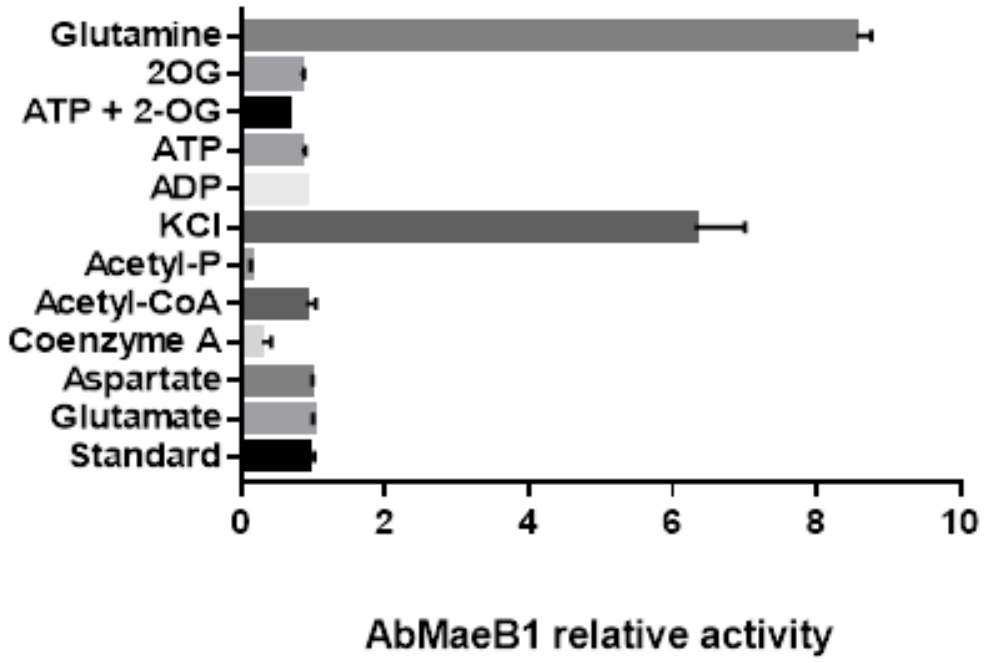

Figure 5 - Regulatory properties of AbMaeB1. ME activity was measured in the presence of 5 mM of L-malate and 0.5 mM of $NADP^+$. Results were plotted as relative activity of standard reaction (1). Average data from duplicate assays ± SD. Effectors are indicated on the *y* axes. KCl 2mM, Acetyl-P 1 mM, Acetyl-CoA 0.6 mM, Coenzyme A 1.5 mM, Aspartate 4.5 mM, Glutamate 100 mM, ADP 1 mM, ATP 1 mM, 2OG 1.5 mM, Glutamine 1.5 mM.

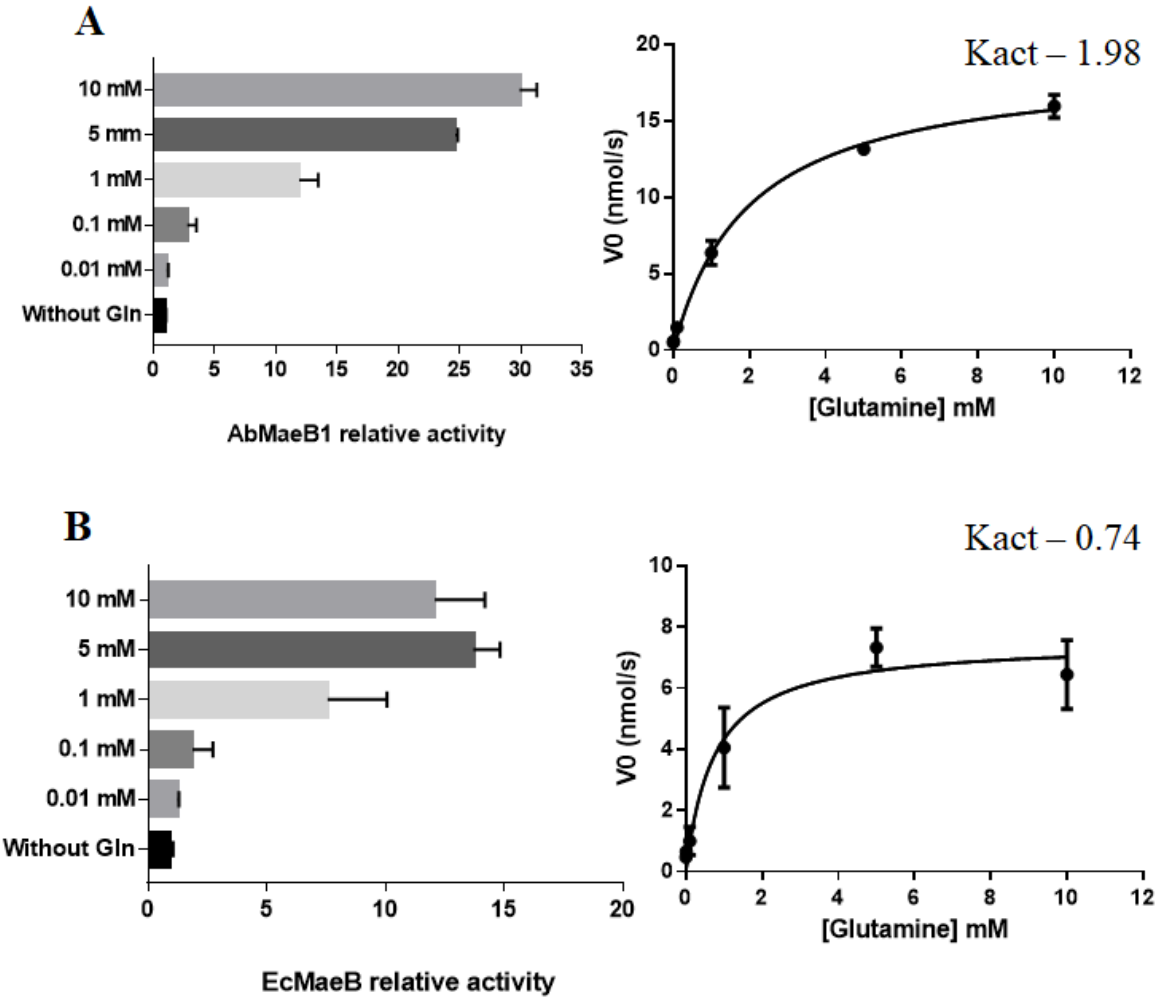

Figure 6 – Effect of different glutamine concentrations in AbMaeB1 (A) and EcMaeB activity (B). ME activity was measured in the presence of 5 mM of L-malate and 0.5 mM of $NADP^+$. The activation constants for glutamine are indicated in the respectives graphs. Results were plotted as relative activity of standard reaction (1). Average data from duplicate assays ± SD.

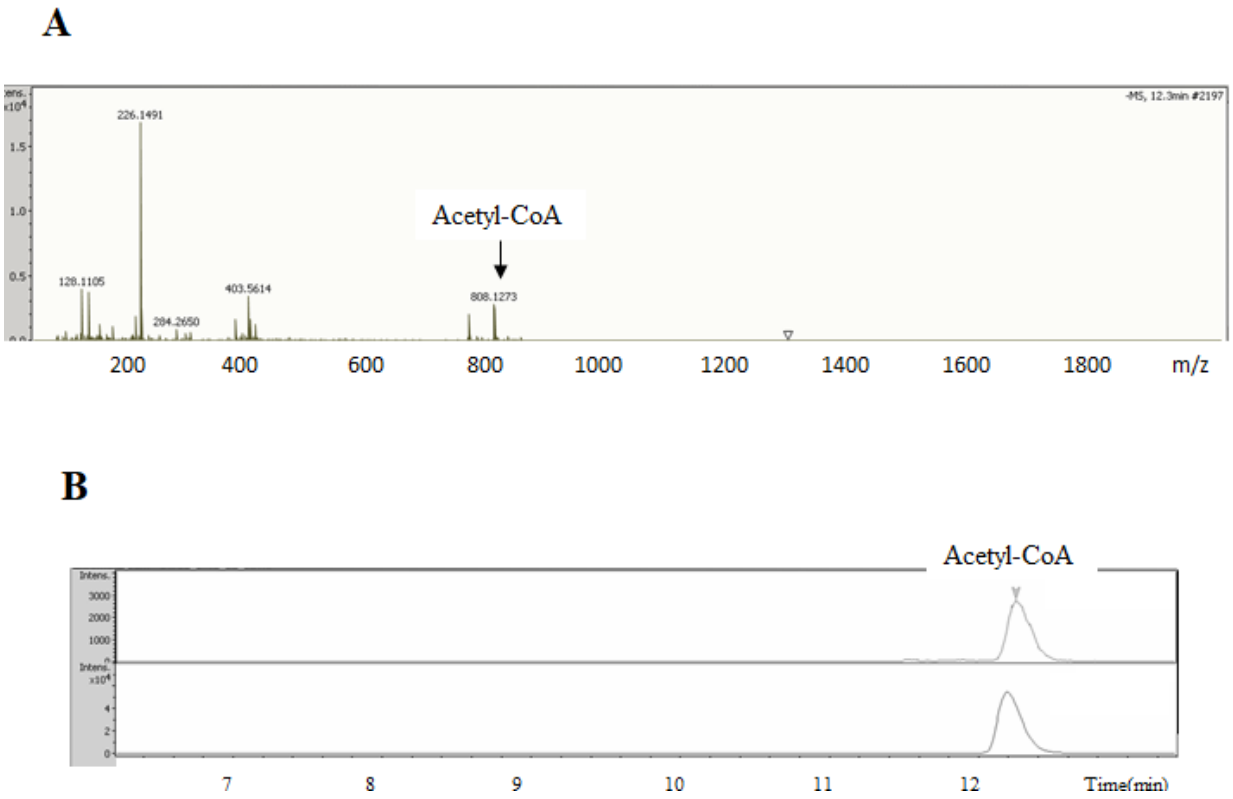

Figure 7 - Formation of Acetyl-CoA by MaeB detected by LC/MS. (A) *A. brasilense* MaeB1 was incubated for 30 min with CoA and Acetyl-P, reactions were quenched and analyzed by LC/MS. A peak with m/z of 808.12 and retention time of 12.2 min was assigned as Acetyl-CoA (theoretical m/z 808.1185, and retention time confirmed by a comercial standard). This peak was not present in control reactions without the enzyme (not shown). (B) *E. coli* MaeB His (EcMaeB His) or untagged MaeB (EcMaeB) were incubated at 30^0C in the presence of CoA and acetyl-P, after 30 min the reactions were analyzed by LC/MS. The extracted ion chromatogram (808.1185 ± 0.01 m/z) is shown with the acetyl-CoA peak indicated by an arrow. The intensity of the peak for His tagged MaeB was one order of magnitude lower than observed with untagged native EcMaeB.

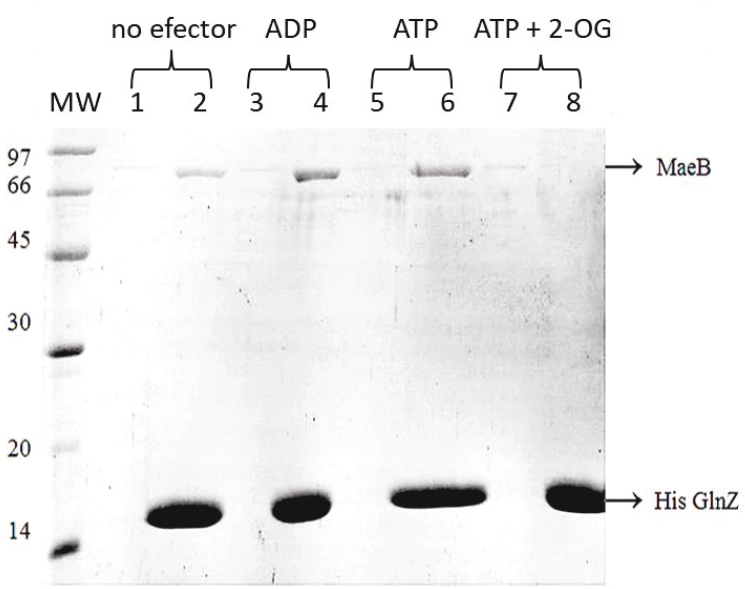

Figure 8 - *In vitro* formation of the GlnZ-MaeB1 complex. Complex formation was assessed by co-precipitation using Ni^{2+} beads. Reactions were performed in the presence of the indicated effectors, ATP and ADP at 1mM and 2-OG 1.5 mM. Binding reactions were conducted in 400 µl of buffer adding the purified proteins MaeB1 all lanes and HisGlnZ (Even lanes). The eluted fractions was subjected to SDS-PAGE, the gel was Coomassie blue stained. MW, molecular weight markers in kDa.

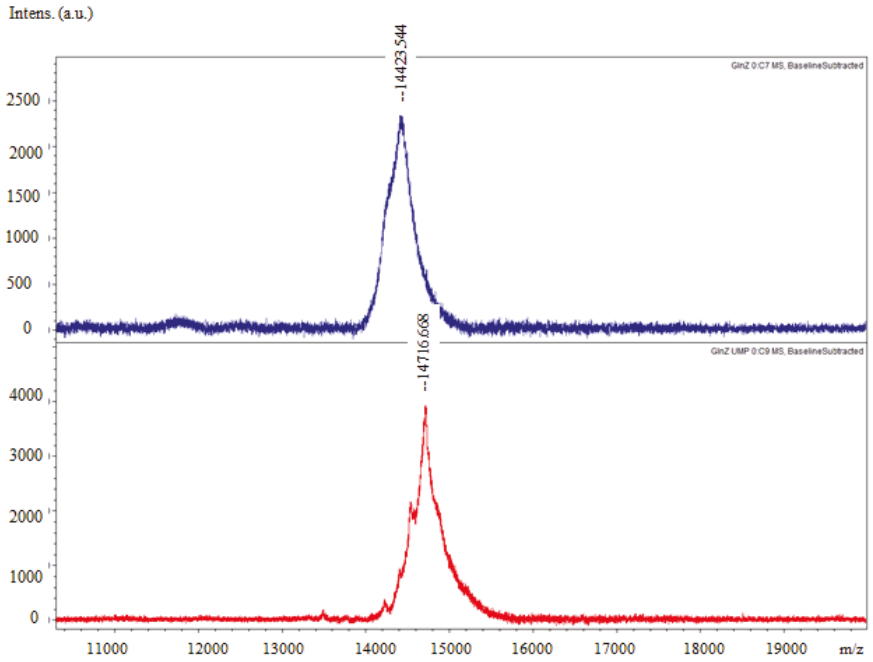

Figure 9 – MALDI-TOF analysis of unmodified (A) and uridylylated (B) GlnZ showing the mass shift corresponding to the UMP group.

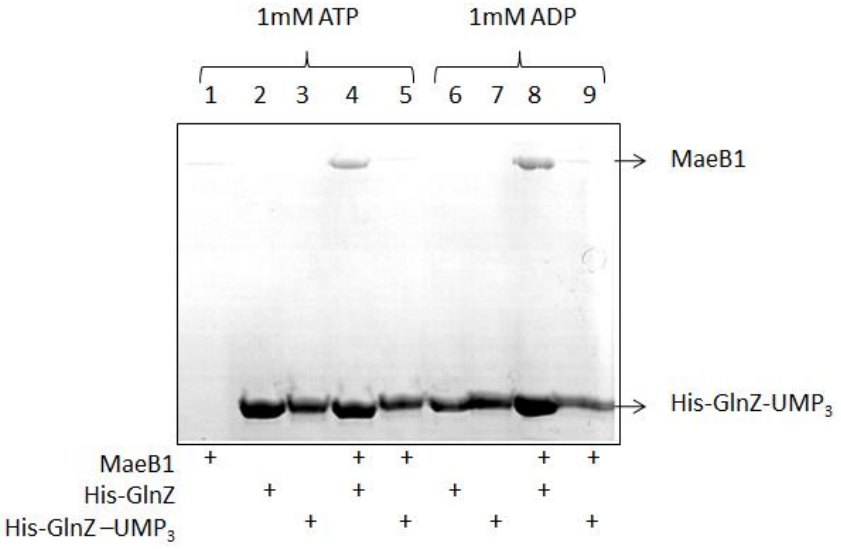

Figure 10 – Fully uridylylated GlnZ-UMP_3 can not interact with MaeB1. Unmodified His-GlnZ (lanes, 2, 4, 6 and 8) or uridylylated His-GlnZ-UMP_3 (lanes, 3, 5, 7 and 9) were bound to Ni^{2+} beads in the presence of the indicated effectors (ATP or ADP). The beads received purified MaeB1 as indicated (Lanes 1, 4, 5, 8 and 9). After extensive washes, bound proteins were eluted with imidazol and subjected to SDS-PAGE, the gels were Coomassie blue stained.

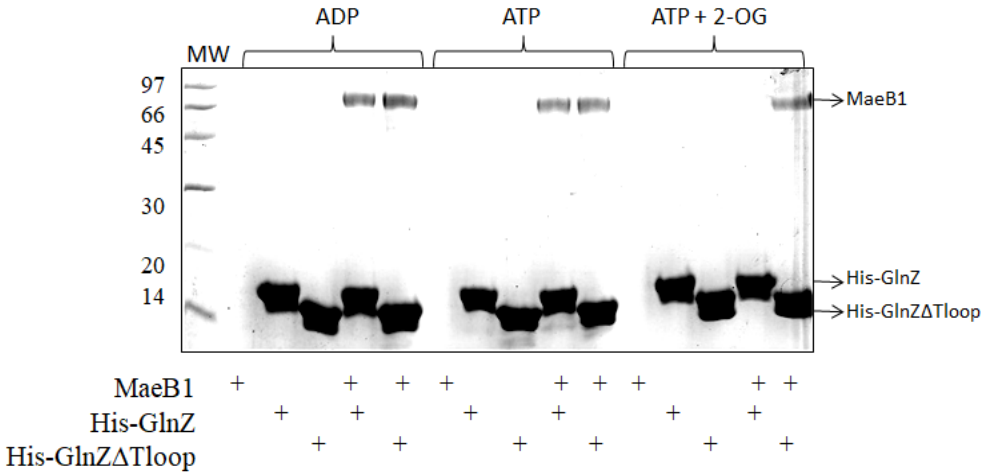

Figure 11 - *In vitro* formation between MaeB1 and the GlnZΔT-loop variant. Complex formation was assessed by co-precipitation using Ni^{2+} beads. Reactions were performed in the presence of the indicated effectors, ATP and ADP at 1mM and 2-OG 1.5 mM. Binding reactions were conducted in 400 µl of buffer adding the purified proteins as indicated in the figure. The eluted fractions was subjected to SDS-PAGE, the gel was Coomassie blue stained. MW, molecular weight markers in kDa.

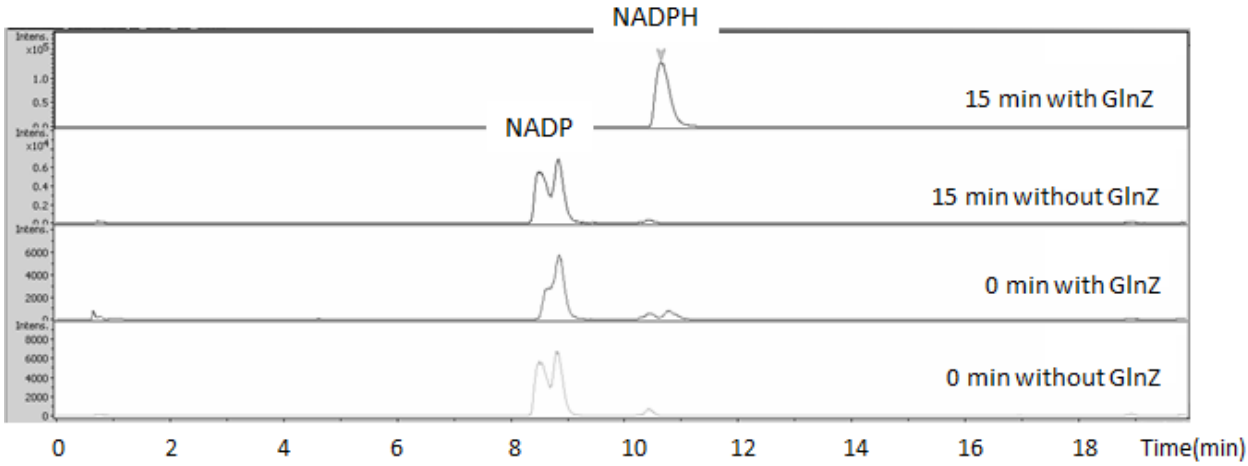

Figure 12 - Formation of NADPH by AbMaeB1 analyzed by LC/MS. The direct malic enzyme reaction was analyzed by LC/MS assays by detecting simultaneously the m/z of the product NADPH and substrate $NADP^+$ (indicated by arrows) after 0 or 15 min of incubation at 30^0C in the presence or absence of excess GlnZ. The chromatogram was generating by extraction the NADPH and $NADP^+$ theoretical mass ± 0.01 m/z. In the presence of GlnZ more NADPH is formed and $NADP^+$ is completely consumed.

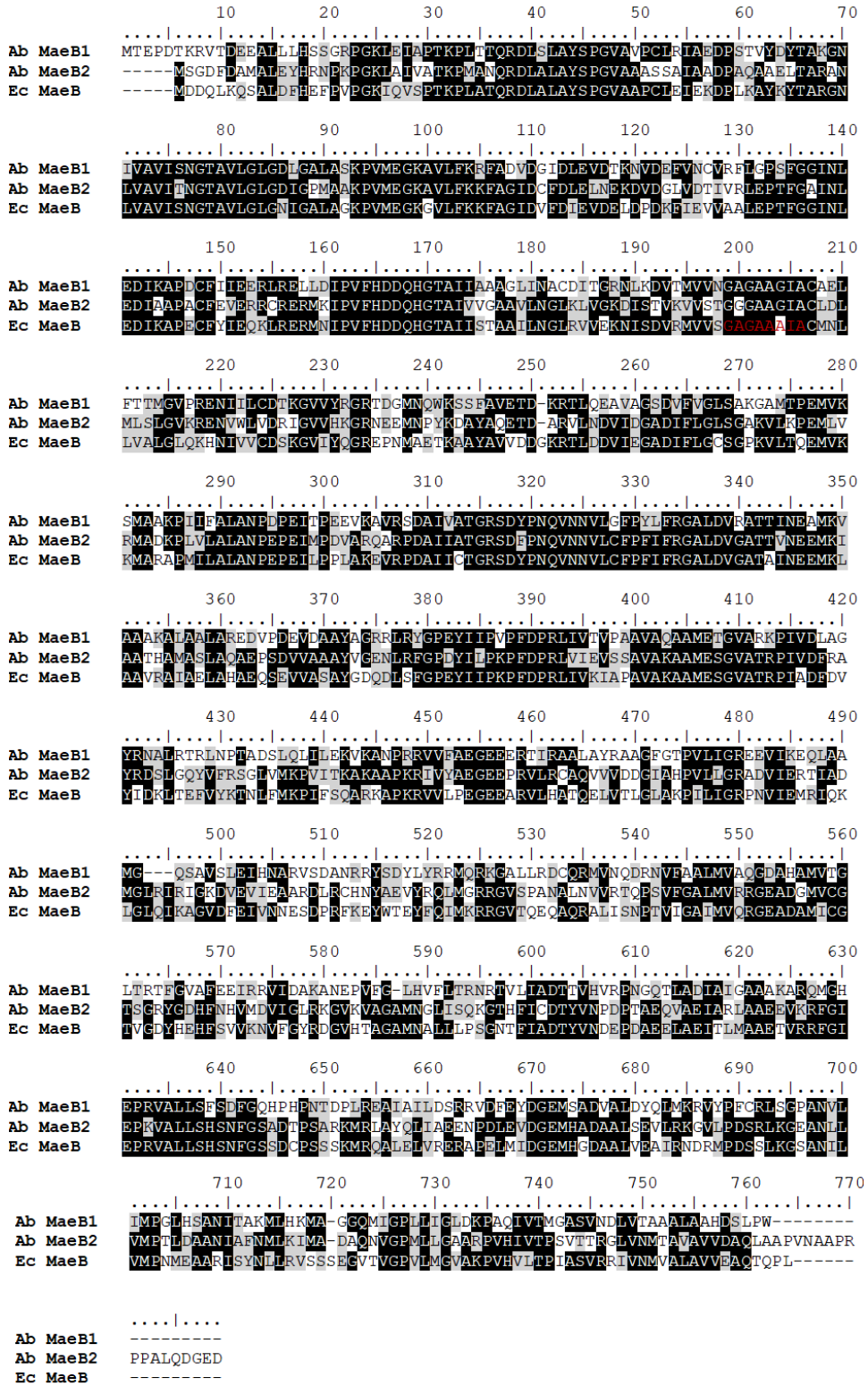

Figure S1 – Sequence alignment of *A. brasilense* MaeB1 (WP_014238784.1), MaeB2 (WP_014198543.1) and *E. coli* MaeB (Uniprot P26616). Sequences were aligned using clustal W. Identical and similar residues are shaded in black and gray, respectively. The putative $NADP^+$ binding site in *E. coli* MaeB are shown in red.


Archaea
Bacteroidetes
A0A256J217 A0A256J217 9EURY NADP-dependent malic enzyme Halorubrum ezzemoulense
A0A256JYT5 A0A256JYT5 9EURY NADP-dependent malic enzyme Halorubrum ezzemoulense
A0A238XWW5 A0A238XWW5 HALC1 Allosteric NADP-dependent malic enzyme Halorubrum chaoviator
A0A256JDG5 A0A256JDG5 9EURY NADP-dependent malic enzyme Halorubrum ezzemoulense
A0A256JKS3 A0A256JKS3 9EURY NADP-dependent malic enzyme Halorubrum ezzemoulense
A0A1I6I242 A0A1I6I242 HALSD Allosteric NADP-dependent malic enzyme Halorubrum sodomense
A0A1G7SC83 A0A1G7SC83 9EURY Allosteric NADP-dependent malic enzyme Halorubrum xinjiangense
A0A256L8W1 A0A256L8W1 9EURY NADP-dependent malic enzyme Halorubrum distributum
A0A0F8CK22 A0A0F8CK22 9EURY NAD-binding malic protein Halorubrum saccharovorum
A0A238UPR9 A0A238UPR9 HALVU Allosteric NADP-dependent malic enzyme Halorubrum vacuolatum
A0A1M5TI75 A0A1M5TI75 9EURY Allosteric NADP-dependent malic enzyme Halobaculum gomorrense
A0A1I0QKZ3 A0A1I0QKZ3 9EURY Allosteric NADP-dependent malic enzyme Halobacterium jilantaiense
A0A1H2V7P5 A0A1H2V7P5 HALVA Allosteric NADP-dependent malic enzyme Haloarcula vallismortis
A0A202E7F8 A0A202E7F8 9EURY NADP-dependent malic enzyme Natronolimnobius baerhuensis
A0A1S8AV17 A0A1S8AV17 9EURY NAD-binding malic protein Haloterrigena saccharevitans
A0A1I1L0Q7 A0A1I1L0Q7 9EURY Allosteric NADP-dependent malic enzyme Halobiforma haloterrestris
A0A1I3L7N6 A0A1I3L7N6 9EURY Allosteric NADP-dependent malic enzyme Natronobacterium gregoryi
A0A0K1IUA6 A0A0K1IUA6 9EURY NAD-binding malic protein Haloferax gibbonsii
A0A1H7PHP6 A0A1H7PHP6 9EURY Allosteric NADP-dependent malic enzyme Haloferax larsenii
A0A0K1IVJ3 A0A0K1IVJ3 9EURY NAD-binding malic protein Haloferax gibbonsii
A0A1H7QNQ5 A0A1H7QNQ5 9EURY Allosteric NADP-dependent malic enzyme Haloferax larsenii
A0A1U7ETD8 A0A1U7ETD8 NATPD Malate dehydrogenase (Oxaloacetate-decarboxylating) Natronomonas pharaonis DSM 2160
A0A1U7EVD8 A0A1U7EVD8 NATPD Malate dehydrogenase (Oxaloacetate-decarboxylating) Natronomonas pharaonis DSM 2160
A0A1M5NWD3 A0A1M5NWD3 9EURY Malate dehydrogenase (Oxaloacetate-decarboxylating)(NADP+) Halobaculum gomorrense
A0A1S8AVK5 A0A1S8AVK5 9EURY NAD-binding malic protein Haloterrigena saccharevitans
A0A1N7E1S9 A0A1N7E1S9 9EURY Allosteric NADP-dependent malic enzyme Natronorubrum thiooxidans
A0A202EB05 A0A202EB05 9EURY NADP-dependent malic enzyme Natronolimnobius baerhuensis
A0A1H7S4K9 A0A1H7S4K9 9SPHI Allosteric NADP-dependent malic enzyme Parapedobacter koreensis
A0A246EUV1 A0A246EUV1 PREIN NADP-dependent malic enzyme Prevotella intermedia
A0A255SML8 A0A255SML8 PREBR NADP-dependent malic enzyme Prevotella bryantii
A0A1Q6HCK2 A0A1Q6HCK2 9BACE NADP-dependent malic enzyme Bacteroides finegoldii
A0A1N7K364 A0A1N7K364 9FLAO Allosteric NADP-dependent malic enzyme Chryseobacterium joostei
A0A1M5VZ59 A0A1M5VZ59 FLAPS Malate dehydrogenase (Oxaloacetate-decarboxylating) (NADP+) Flavobacterium psychrophilum
A4BX05 A4BX05 9FLAO Putative NADP-dependent malic enzyme Polaribacter irgensii 23-P
A0A1I4XMD1 A0A1I4XMD1 9FLAO Allosteric NADP-dependent malic enzyme Salegentibacter flavus
A0A1H7QWR9 A0A1H7QWR9 9FLAO Allosteric NADP-dependent malic enzyme Maribacter orientalis
A0A1M6KJT7 A0A1M6KJT7 9FLAO Allosteric NADP-dependent malic enzyme Pseudozobellia thermophila
A0A161PCD6 A0A161PCD6 BDEBC NAD-binding malic protein Bdellovibrio bacteriovorus
A0A143NKH3 A0A143NKH3 9RHIZ Malic enzyme Mesorhizobium ciceri
A0A271LJ70 A0A271LJ70 9RHIZ NADP-dependent malic enzyme Mesorhizobium temperatum
A0A0K2VYF5 A0A0K2VYF5 MESPL Putative fused malic enzyme oxidoreductase phosphotransacetylase Mesorhizobium plurifarium
A0A1I4BG69 A0A1I4BG69 9RHIZ Allosteric NADP-dependent malic enzyme Mesorhizobium albiziae
AAB82460.1 NADP-dependent malic enzyme TME Sinorhizobium meliloti
A0A1L5NDX5 A0A1L5NDX5 9RHIZ NADP-dependent malic enzyme Rhizobium gallicum
A0A137Y0I9 A0A137Y0I9 OCHAN Malic enzyme Ochrobactrum anthropi
Ab MaeB1
A0A074ME01 A0A074ME01 9SPHN Malic enzyme Erythrobacter litoralis
A3WF17 A3WF17 9SPHN Phosphate acetyltransferase Erythrobacter sp. NAP1
A0A147HTZ3 A0A147HTZ3 9SPHN Malic enzyme Sphingomonas sanguinis
A0A147IL44 A0A147IL44 9SPHN Malic enzyme Sphingomonas yabuuchiae
A0A031JGR6 A0A031JGR6 SPHPI NADP-dependent malic enzyme Sphingomonas paucimobilis
A0A0A1Y7L6 A0A0A1Y7L6 9SPHN Malic enzyme Sphingomonas sp. BHC-A
A0A0M3AUT2 A0A0M3AUT2 9SPHN Malic enzyme Sphingobium chungbukense
A0A292ZCG1 A0A292ZCG1 SPHSA NADP-dependent malic enzyme Sphingobium fuliginis
A0A291N0R9 A0A291N0R9 SPHYA NADP-dependent malic enzyme Sphingobium yanoikuyae
A0A154L8X1 A0A154L8X1 9PROT Malic enzyme Thalassospira lucentensis
A1WVU3 A1WVU3 HALHL Allosteric NADP-dependent malic enzyme Halorhodospira halophila SL1
A0A1I2X2L2 A0A1I2X2L2 9RHOB Malate dehydrogenase (Oxaloacetate-decarboxylating)(NADP+) Paracoccus aminovorans
A3VAX4 A3VAX4 9RHOB NAD dependent malic enzyme Maritimibacter alkaliphilus HTCC2654
Ab MaeB2
A0A060DKQ9 A0A060DKQ9 AZOBR Malic enzyme Azospirillum brasilense
A0A090MPP5 A0A090MPP5 AFIFE NAD-dependent malic enzyme Afipia felis
A0A2A6NFH7 A0A2A6NFH7 9BRAD NADP-dependent malic enzyme Bradyrhizobium sp. C9
A0A011UHF3 A0A011UHF3 OCHAN Malic enzyme Ochrobactrum anthropi
WP_100673238.1 NADP-dependent malic enzyme DME Sinorhizobium meliloti
A0A154I964 A0A154I964 RHILE Malic enzyme Rhizobium leguminosarum
A0A0F4FTH8 A0A0F4FTH8 RHIRD NADP-dependent malic enzyme Agrobacterium tumefaciens
A0A0T7G293 A0A0T7G293 RHIGA NADP-dependent malic enzyme Neorhizobium galegae bv. officinalis
A0A0T7H5U4 A0A0T7H5U4 RHIGA NADP-dependent malic enzyme Neorhizobium galegae bv. officinalis
A0A1B8Q8L7 A0A1B8Q8L7 9GAMM Malic enzyme Moraxella atlantae
A0A1V4GZS3 A0A1V4GZS3 MORLA NADP-dependent malic enzyme Moraxella lacunata
A0A0A8TIU3 A0A0A8TIU3 ACIBZ NADP-dependent malic enzyme Acinetobacter bereziniae
A0A0D7LEU2 A0A0D7LEU2 CITFR Malic enzyme Citrobacter freundii
A0A140T2W6 A0A140T2W6 CITAM Malic enzyme Citrobacter amalonaticus
Ec MaeB
A0A1V2Z4Q4 A0A1V2Z4Q4 9ENTR NADP-dependent malic enzyme Leclercia adecarboxylata
A0A0F0YIK5 A0A0F0YIK5 9ENTR Malic enzyme Enterobacter kobei
A0A263VU70 A0A263VU70 ENTCL NADP-dependent malic enzyme Enterobacter cloacae
A0A170T555 A0A170T555 KLEPN NADP-dependent malic enzyme Klebsiella pneumoniae
A0A1V2BGA7 A0A1V2BGA7 RAOTE NADP-dependent malic enzyme Raoultella terrigena
A0A209AC97 A0A209AC97 YERKR NADP-dependent malic enzyme Yersinia kristensenii
A0A147FYZ6 A0A147FYZ6 9GAMM Malic enzyme Pantoea dispersa
A0A1X1EWP4 A0A1X1EWP4 PANCY NADP-dependent malic enzyme Pantoea cypripedii
A0A094TK08 A0A094TK08 PROVU NADP-dependent malic enzyme Proteus vulgaris
A0A071MD72 A0A071MD72 9BURK Malic enzyme Burkholderia cenocepacia
A0A103U9D4 A0A103U9D4 BURCE Malic enzyme Burkholderia cepacia
A0A1M6X7X7 A0A1M6X7X7 9BURK Malate dehydrogenase (Oxaloacetate-decarboxylating)(NADP+) Paraburkholderia terricola
A0A1H6YWR6 A0A1H6YWR6 9BURK Malate dehydrogenase (Oxaloacetate-decarboxylating)(NADP+) Paraburkholderia tropica
A0A0A1FCS4 A0A0A1FCS4 9BURK NADP-dependent malic enzyme Collimonas arenae
A0A127QVB0 A0A127QVB0 9BURK Malic enzyme NAD binding domain protein Collimonas pratensis
A0A0S4TNI3 A0A0S4TNI3 RALSL Malic enzyme Ralstonia solanacearum
A0A1K0I9W4 A0A1K0I9W4 CUPNE Putative fused malic enzyme oxidoreductase putative phosphotransacetylase Cupriavidus necator
A0A104TWN9 A0A104TWN9 9BURK Malate dehydrogenase Burkholderia ubonensis
A0A239SQW9 A0A239SQW9 9BURK NADP-dependent malic enzyme Pandoraea sputorum
A0A0H3LJG4 A0A0H3LJG4 BORBR NADP-dependent malic enzyme Bordetella bronchiseptica RB50
A0A125NZK0 A0A125NZK0 9BURK NADP-dependent malic enzyme Variovorax sp. WDL1
A0A1C0UHQ0 A0A1C0UHQ0 RALSL Malate dehydrogenase Ralstonia solanacearum
A0A0A8BRZ0 A0A0A8BRZ0 9BURK Malate dehydrogenase Pandoraea pulmonicola
A0A0P0KZ79 A0A0P0KZ79 9BURK Malic enzyme Burkholderia plantarii
A0A2A7S8J1 A0A2A7S8J1 BURGA NADP-dependent malic enzyme Burkholderia gladioli
A0A269S3V8 A0A269S3V8 9BURK NADP-dependent malic enzyme Burkholderia ubonensis
A3NDM9 A3NDM9 BURP6 NADP-dependent malic enzyme/phosphate acetyl/butyryltransferase family protein Burkholderia pseudomallei 668
A0A0D0HXN1 A0A0D0HXN1 STEMA Malic enzyme Stenotrophomonas maltophilia
A0A0A6WSE4 A0A0A6WSE4 XANCH Malic enzyme Xanthomonas campestris pv. phaseoli
A0A1V9GTD2 A0A1V9GTD2 9XANT Malic enzyme Xanthomonas phaseoli pv. dieffenbachiae

Figure S2 - Phylogenetic analysis of selected Malic-PTA fused enzymes. The sequences of malic enzymes with fused PTA domain (malic enzyme with phosphate acetyl/butaryl transferase domain PIRSF036684) from diverse taxonomic groups were retrieved from PIRSF server (http://pir.georgetown.edu/cgi-bin/tax_phy_adv.pl?SGid0=SF036684&p=SF036684). Sequences for *A. brasilense* MaeB1 (AbMaeB1), *A. brasilense* MaeB2 (AbMaeB2), *E. coli* MaeB (EcMaeB), *S. meliloti* DME MaeB (WP 100673238.1) *and S. meliloti* TME MaeB (AAB82460.1) are indicated by red rectangles. Sequences were aligned using Clustal W. The phylogentic tree was constructed by Neighbor-joining using MEGA 7. All positions containing gaps and missing data were eliminated from the dataset. Bootstrap values were adjusted to 1000 replicates. Sequences from Archaea and Bacteroidetes formed separeted clusters as indicated.

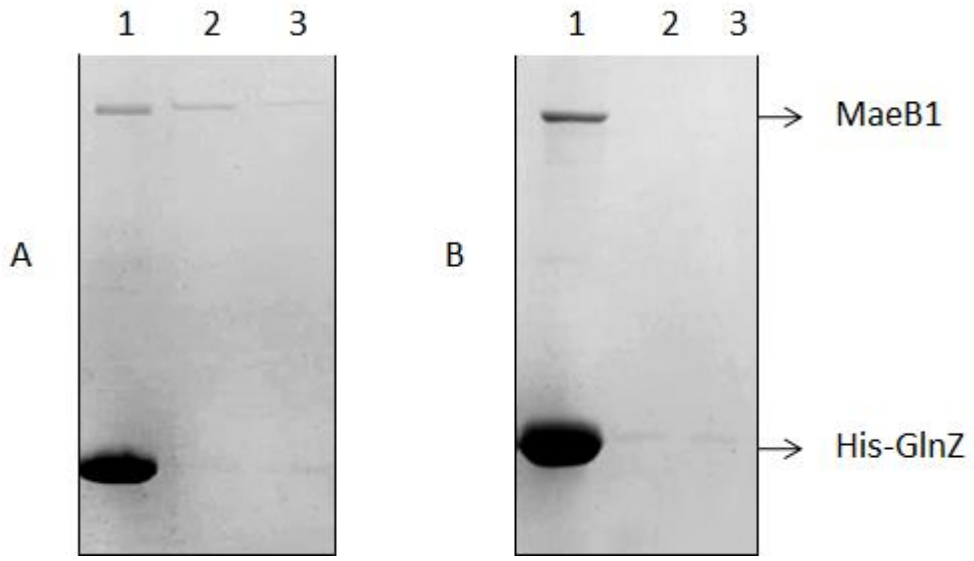

Figure S3 - *In vitro* dissociation of the GlnZ-MaeB1 complex. The MaeB1-GlnZ complex was mobilized in Ni^{2+} beads in the presence of 1 mM ATP (A) or ADP (B). The beads were subjected to two washing steps in buffer containing the indicated nucleotides plus 1.5 mM 2-OG (lanes 2 and 3 represent the fractions eluted from the two consecutive washes with 2-OG). This was followed by a final elution step with Imizadol (Lanes 1). The eluted fractions were subjected to SDS-PAGE, the gels were Coomassie blue stained.

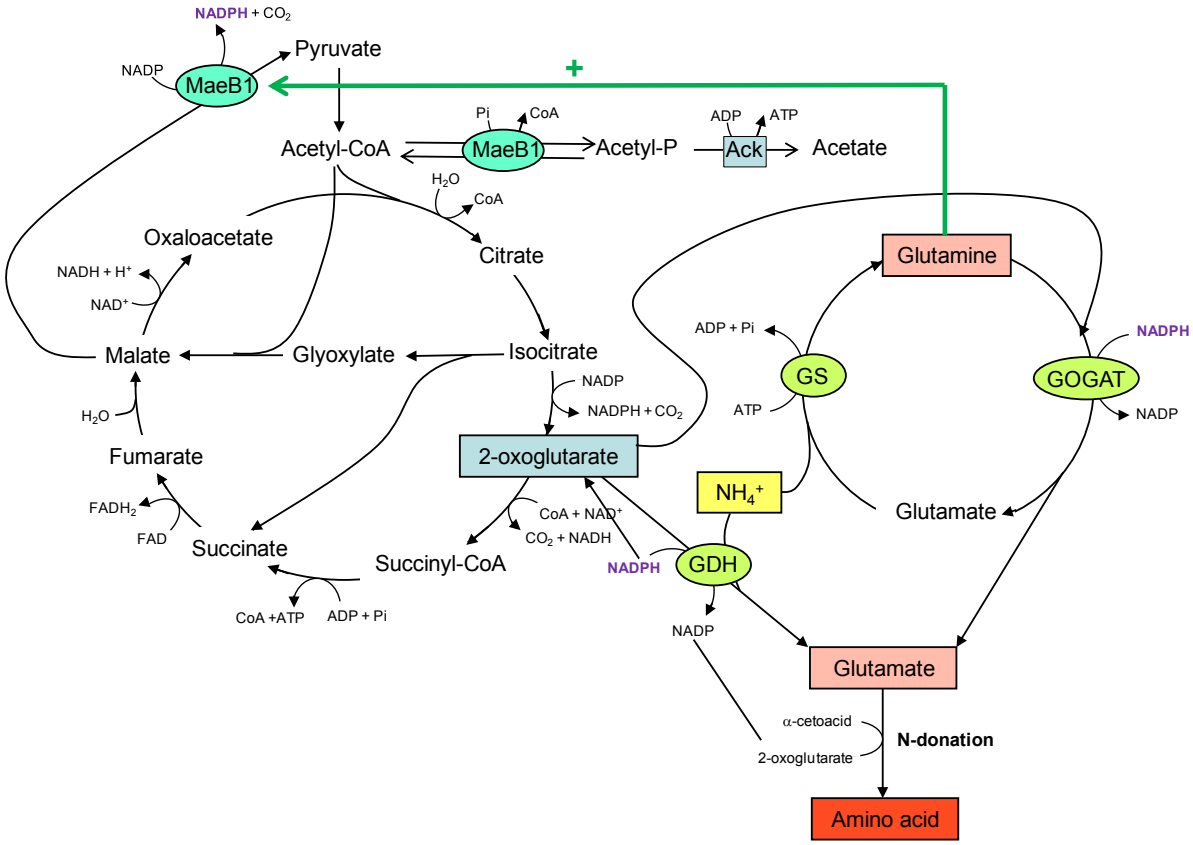

Figure S4 - Schematic representation of TCA cycle, nitrogen assimilation and effect of glutamine in the activity of malic enzyme of MaeB. This diagram also illustrates the participation of PTA activity of MaeB on carbon distribution. Note that the reversibility of the PTA activity of MaeB was not experimentally confirmed (Figure generated by Luciano Huergo).

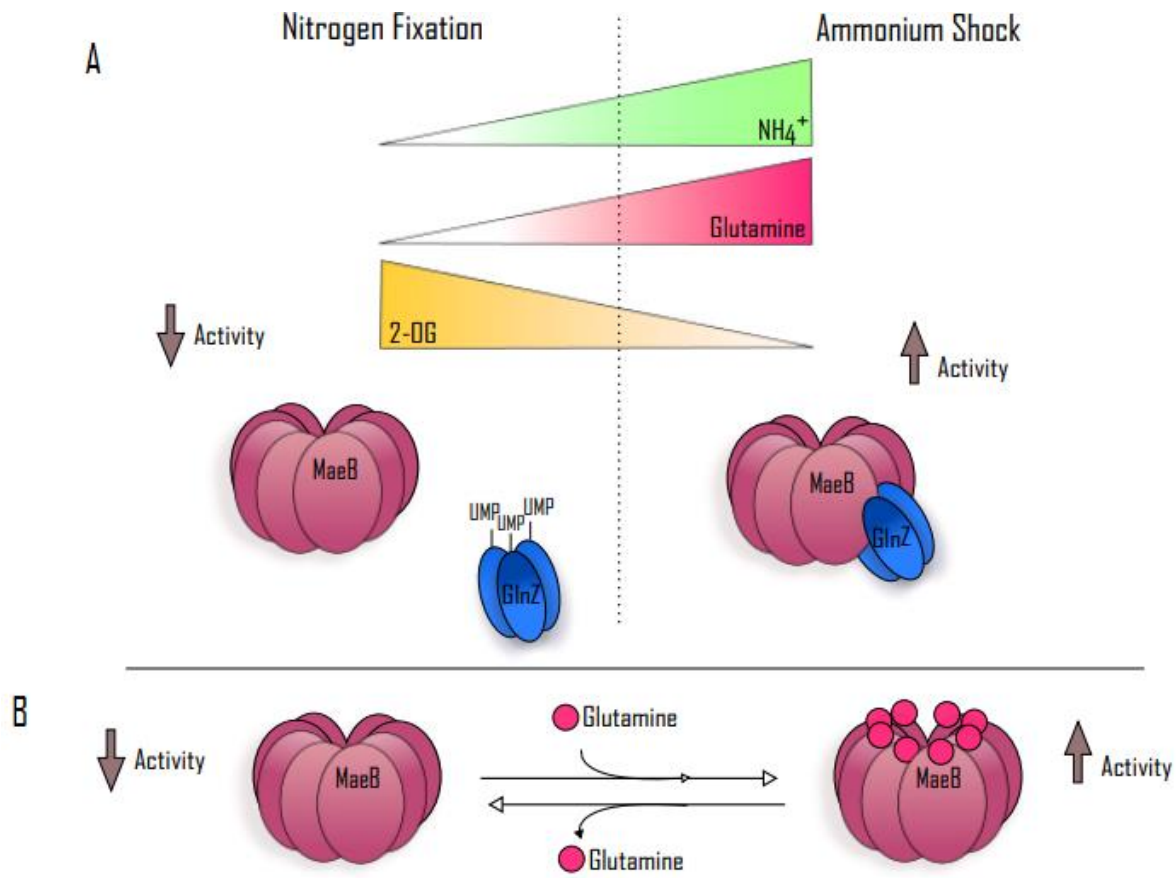

Figura S5 - Model for the role of GlnZ protein and glutamine in the regulation of MaeB in response to ammonium. Up and down arrows indicate the activity of Maeb. (Figure generated by Fernanda Gravina).

4. CAPÍTULO II

The signal transduction GlnZ protein interacts with N-acetylglutamate kinase in *Azospirillum brasilense*

Introduction

N-acetylglutamate kinase (NAGK, E.C. 2.7.2.8) takes part in the biosynthesis of arginine, by promoting the phosphorylation of the –COOH group of N-acetylglutamate and forming N-acetylglutamyl phosphate (Cunin *et al*., 1986). In many organisms, such as in *Escherichia coli,* the protein is organized as a dimer, in others, like *Synechoccocus elongatus*, the subunits of this enzyme form a hexamer. The dimeric NAGKs are not sensitive to arginine (Fernández-Murga and Rubio, 2008; Slocum, 2005), while the hexameric forms are able to bind arginine and this amino acid acts as a feedback inhibitor of NAGK controlling the arginine biosynthesis pathway (Cunin *et al*., 1986).

Arginine is a form of nitrogen storage and it is utilized in synthesis of proteins, arginine polymers and polyamines (Lee *et al.*, 2009; Maheswaran et al., 2006; Slocum, 2005; VanEtten *et al.,* 1963). In organisms were NAGK is inhibited by arginine (Cunin *et al.*, 1986; Slocum, 2005; Xu *et al.,* 2007), a regulatory mechanism is present to allow arginine accumulation. In cyanobacteria and plants it was shown that the regulatory PII proteins interact with NAGK thereby relieving the arginine feedback on NAGK under certain conditions (Maheswaran *et al.,* 2004).

The PII proteins are homotrimers of 12-13 kDa subunits, belonging to a well-conserved family of signaling proteins presented in all Bacteria, Archaea and cloroplasts from red algae and plants (Arcondéguy *et al.*, 2001). In Proteobacteria it is common to find more than one paralog gene of PII protein. In the diazotrophic bacterium *Azospirillum brasilense*, two PII proteins were identified, namely, GlnZ and GlnB.

The PII protein structure is very conserved, each PII subunit contains three main structures: the loops –T, -C and –B (Arcondéguy *et al.,* 2001). The loop T has an important role in interaction between PII and other proteins (Arcondéguy et al., 2001). The three loops together form the binding sites of the effector molecules, ATP, ADP and 2-OG (Truan *et al.,* 2010). Since PII binds these molecules, these proteins can sense the nitrogen/carbon and energy levels (Gerhardt, 2012; Huergo *et al.,* 2012; Zhang *et al.,* 2013; Zhang and Ye, 2014).

In some organisms PII proteins are subject to a cycle of reversible posttranslational modification. In Proteobacteria, the PII proteins are subjected to reversible uridylylation in the T-loop (Merrick, 2014) and this modification is controlled by the intracellular glutamine levels (Araújo *et al*., 2008; Jiang and Ninfa, 1999).

The NAGK enzyme of *A. brasilense* was recently identified as a potential target of GlnZ (Gerhardt *et al.,* unpublished). The complex formation between PII and the hexameric form of NAGK has already been well described in cyanobacterias and plants (Ferrario-Méry *et al.,* 2006; Maheswaran *et al.,* 2004) and it was speculated that these complex would be restricted to these class of organisms. Here we show the partial purification of *A. brasilense* NAGK and complex formation with GlnZ.

Experimental procedures

Bacterial strains, cloning and molecular biology methods

The Table 1 shows the bacterial strains used in this work. Isolation of plasmid DNA, gel electrophoresis, bacterial transformation and cloning were performed as described (Sambrook *et al.,* 1989). Enzymes were obtained from commercial sources and used according to the manufacturers' instructions. DNA sequencing was performed using dye-labeled terminators in an automated DNA sequencer ABI 3500 from Applied Biosystems.

The *argB* gene from *Azospirillum brasilense* FP2 was amplified by PCR using genomic DNA as template, *Pfu*X7 DNA polymerase (Nørholm, 2010) and the primers: 5' TCCATCATATGCAGAACACGACCCGC 3' in combination with 5' CGCTTGGATCCTCACTCGCGGCCGATCA 3' (Restriction sites for *Nde*I and *Bam*HI are underlined). The products generated by PCR were digested by the restriction enzymes *Nde*I and *Bam*HI and ligated into pET29a, previously digested with the same enzymes. The plasmids containing the AbNAGK were isolated and submitted to sequencing to confirm the identity of the gene. The plasmid was named pGANAGK (pET29a expressing NAGK).

The plasmid pMSA4dellloopT expressing the version of GlnZ containing a deletion on the T-loop (GlnZΔ42-54) (Scarduelli, 2010) was used to generate a N-terminal His-tagged version of this protein, by subcloning *glnZ Nde*I and *Bam*HI fragment of pMSA4dellloopT into the *Nde*I and *Bam*HI sites of pET28a. The resulting plasmid was named pGAHisGlnZΔ*loop*.

Expression and purification of proteins

Overexpression of the AbNAGK

The pGANAGK plasmid was transformed into *E. coli* BL21 (DE3). The cells were grown to a D.O of 0.6 in LB and the addition of 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) initiated the overexpression of NAGK at 37ºC for 3 h. The cells were centrifuged (2,240 g, 10 minutes, 4ºC) and kept at -40ºC until use. Cells were resuspended in 50 mM Tris-HCl pH 7.5, 50 mM NaCl and lysed by sonication. The solube fraction was collected after centrifugation at 20,000 g, 4ºC, 10 min.

Binding of NAGK to different matrices

In order to determine the best resine for NAGK purification we tested five different chromatographic matrices (Q FF, DEAE FF, CM FF, SP FF and Heparin HP (GE Healthcare)). 50 mM Tris-HCl pH 7.5, 50 mM NaCl was used as buffer A for cell lysis, column equilibration and washes. One ml of each matrix was aliquoted and washed with buffer A, then 500 µl of the soluble fraction of NAGK was incubated with each matrix for 10 minutes, and an aliquot was collected. Two washes with buffer A and a final elution with buffer B (50 mM Tris-HCl pH 7.5; 1M NaCl) was carried out and fractions of 10 µl were collected and applied in a 12,5% SDS-PAGE to analysis.

Purification of AbNAGK

The protein was overexpressed as described above. The cell pellet was ressuspended in buffer A and the cells were disrupted by sonication, the solution was then submitted to centrifugation (20,000 g, 4ºC, 30 min) in order to remove the unbroken cells and the cell debris. The supernatant was loaded on a 5 ml DEAE column, the column was washed with buffer A and the bound protein was eluted with a NaCl gradient (50-1000 mM). The elution was collected in 1 ml fractions and verified by SDS-PAGE. Fractions containing NAGK were selected, pooled and submitted to dialysis in dialysis Buffer (Tris HCl 50 mM pH 8.0, NaCl 100 mM, glycerol 10% (v/v)), and stored in aliquots at -40ºC until use. Proteins concentration was determined by the Bradford assay using bovine serum albumin (BSA) as standard.

Purification of GlnZ

The expression and purification of His-GlnZ was carried on as described in *(Moure et al.,* 2012). His-GlnZΔT*loop* was purified using the same protocol.

Purification of GlnD

The GlnD protein from *Escherichia coli* was purified according to Rodrigues (2017).

In vitro uridylylation of His-GlnZ

The *in vitro* uridylylation of His-GlnZ was performed as described in Bonatto *et al.* (2007). The buffer used was composed of 100 mM Tris–HCl pH 7.5, 100 mM KCl and 25 mM $MgCl_2$. The reaction mix contained 100 µM His-GlnZ (considered as monomer), 1 mM ATP, 2 mM UTP, 5 mM 2-ketoglutarate, 1 µM of purified *E. coli* GlnD and was incubated overnight at 37ºC. The reaction was ceased by heating at 60ºC for 15 min to GlnD denaturation which was further removed by centrifugation. The uridylylation was confirmed by Native-PAGE and MALDI-TOF mass spectrometry.

Gel filtration chromatography

In order to predict the molecular mass and oligomeric state of NAGK, the purified protein was submitted at analytical gel filtration chromatography on a Superose 12 10/300 column (GE Healthcare) using Tris-HCl 50 mM pH 8, NaCl 100 mM and glycerol 5 % (v/v) as buffer. The column was calibrated with the following molecular mass markers from Bio-Rad: Gama-globulin (158 kDa); ovalbulmin (44 kDa) and myoglobin (17 kDa). The runs were performed at room temperature at a flow rate of 0.5 ml per minute.

Protein co-purification using magnetic beads

The *in vitro* interaction assays were perfomed using Ni^{2+} Magnetic beads (Promega) as described in Huergo *et al*. (2007), with modifications. The buffer used in the reactions was composed of 50 mM Tris-HCl pH 8, 50 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 20 mM imidazole and Nonidet 10%, in the presence or absence of effectors as indicated in each experiment.

Four microliters of beads were equilibrated by three washes with 200 µl of buffer. Binding reactions were performed in 400 µl of buffer by adding purified proteins (20 ug of

tagged protein and 50 ug of the untagged protein). After 15 minutes, the beads were washed 3 times with 200 µl of buffer. Then, the samples were mixed with sample buffer and analyzed by SDS-PAGE.

Results and Discussion

NAGK occurs in the arginine-insensitive or arginine-inhibitable form, depending on the organism (Fernández-Murga *et al*., 2004). Crystal structures of NAGK show that the dimeric forms, like in *E.coli* (Gil-Ortiz *et al.,* 2003; Ramón-Maiques *et al.,* 2002), are arginine-insensitive and the hexameric forms (*Pseudomonas aeruginosa, Thermotoga maritima* and *Synechoccocus elongatus*) are arginine-sensitive (Llácer *et al.,* 2007; Ramón-Maiques *et al.,* 2006). Several studies have shown that the arginine-sensitive forms of NAGK from cyanobacteria, algae and plants are responsive to the PII signaling protein (Beez *et al.*, 2009; Chellamuthu *et al.,* 2014; Lapina *et al.,* 2018; Minaeva *et al.,* 2015). When the intracellular nitrogen level is high, PII binds to NAGK and releases it of arginine inhibition, allowing the arginine synthesis even when arginine concentrations is high *(*Burillo *et al., 2004;* Heinrich *et al.*, 2004; Llácer *et al.,* 2007; Sugiyama *et al.*, 2004).

Despite these studies, there are no investigations regarding the occurrence of this interaction in non-photosynthetic bacteria. Moreover, in LC/MS/MS assays (Gerhardt, 2015)), *A. brasilense* NAGK (AbNAGK) appears as a potential new target of GlnZ in *A. brasilense*. In order to confirm the NAGK-GlnZ interaction in *A. brasilense* we first establish a purification protocol for AbNAGK. We evaluate the affinity of AbNAGK for different ion exchange matrices (Q FF, DEAE FF, CM FF, SP FF and Heparin HP). AbNAGK was able to bind to the anion exchangers matrices DEAE and Q (Fig.1). The DEAE matrix was used for large scale purification, AbNAGK was eluted using a linear NaCl gradient generating a partially purified preparation (Fig.2 A and B).

There is no information about the AbNAGK oligomerization. Blast analysis indicated that AbNAGK is more similar to the hexameric NAGK from *Synechoccocus sp.* (52% identical and 69% similar), than to the dimeric NAGK from *E.coli* (32% identical and 51% similar) (Fig. 3). In order to know more about AbNAGK oligomerization state, we performed an analytical gel filtration chromatography with the partially purified AbNAGK using a superose 12 column. The AbNAGK was distributed into 3 peaks, the first eluting at void volume suggesting the

presence of aggregates of NAGK. The second and more intense had a calculate mass of 210 kDa which fits with the calculated mass for a NAGK hexamer (33 kDa x 6 = 198 kDa). A less intense peak with a mass corresponding to 39.8 kDa, equivalent to monomeric form of AbNAGK, was also detected (Fig. 4). These data suggest that AbNAGK is predominantly arranged as a hexamer.

Different arginine biosynthesis pathways are present in nature. In organisms encoding arginine insensitive NAGK such as *E. coli*, the acetyl-ornithine intermediate is processed via hydrolyzes. On the other hand, in organisms encoding arginine-inhibitable NAGK such as *S. elongatus*, the acetyl-ornithine intermediate is processed by transacetylation (Fernández-Murga *et al*., 2004; Gil-Ortiz *et al.,* 2010; Slocum, 2005). The arginine biosynthesis route predicted by KEGG suggests that *A. brasilense* processes acetyl-ornithine by transacetylation thereby reinforcing the similarities to the pathway present in cyanobacteria, algae and plant and possible conserved regulation by PII.

The interaction between AbNAGK and PII proteins were investigated using co-precipitation assays in Ni^{2+} magnetic beads. NAGK was incubated with His-GlnB or His-GlnZ in the presence of different combinations of PII effectors (without effector, ATP 1 mM, ADP 1 mM and ATP 1 mM + 2-OG 1.5 mM). No signal of co-precipitation was visualized with His-GlnB (data not shown). On the other hand, we detected a positive interaction between NAGK and GlnZ in the presence of ATP or ADP as effectors (Fig. 5). No signal of NAGK in the elution was observed when using ATP+2OG, suggesting that 2-OG destabilizes the complex.

The NAGK-PII complex destabilization by 2-OG is well described in cyanobacteria and plants suggesting conservation in the mode of complex regulation. However, the positive effect of ADP is intriguing as in all interaction between PII and NAGK documented to date only ATP acts as a positive effector (Fokina *et al*., 2010; Lapina *et al.*, 2018; Llácer *et al.,* 2007) and this is supported by structural analysis showing that ADP binding to PII does not allow complex formation with NAGK due to structural constrains in the T-loop (Llácer *et al.,* 2007).

When we performed co-precipitation assays between NAGK and His-GlnZ-UMP_3, no interaction was observed (Fig. 6) suggesting the involvement of the GlnZ T-loop in the interaction as described for other NAGK-PII complexes previously. Given that modified GlnZ accumulate under a high nitrogen regime, complex formation between NAGK-GlnZ is likely to relief arginine inhibition of AbNAGK allowing arginine to accumulate and to act as substrate for synthesis of nitrogen rich compounds such as polyamines (Caldovic and Tuchman, 2003; Llácer *et al.,* 2008).

Co-precipitation assays were performed using NAGK and a GlnZ variant carrying a T-loop deletion (His GlnZΔT*loop*). No complex formation could be detect under the conditions tested (Fig. 7) suggesting a pivotal role of the GlnZ T-loop in complex formation. This is in agreement with Llácer *et al.* (2007), in their work, they show that one of the points of interaction between PII and NAGK in *S. elongatus* is the distal region of the T loop, which plays an essential role in anchoring PII on NAGK.

Complex formation between PII and NAGK is conserved in cyanobacteria, algae and plants (Beez *et al.,* 2009; Chellamuthu *et al.,* 2014; Lapina *et al.,* 2018; Minaeva *et al.,* 2015). The identification of this interaction in *A. brasilense* expands for the first time the NAGK-PII interaction to a non-phototrophic organism supporting that this protein complex emerged in an ancient common ancestor and was kept highly conserved during evolution.

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Tables and Figures

Table 1 - Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Source/reference
E. coli		
BL21 (λDE3)	*hsdS*, gal(*λclts 857 ind1 Sam7 nin5 lacUV5*-T7 gene 1)	Sambrook *et al.*, 2001
DH10B	Sm^r ; $F^{'}$ [*pro*AB^+ *lacZ*M15]	Invitrogen
A. brasiliense		
FP2	Nal^rSm^r Wild type strain	
SP7	Nif+	Pedrosa e Yates , 1984
Plasmíds		
pET28a	Km^r. Expression vector T7 promoter	Novagen
pET29a	Km^r. Expression vector T7 promoter	Novagen
pMSA4ΔloopT	Km^r . Expresses the *A. brasilense* GlnZ with deletion of T loop (Δ42-54). T7 promoter.	Scarduelli, 2010.
pGAHisGlnZΔ*loop*	Km^r (pET28a). Expresses the *A. brasilense* GlnZ with deletion of T loop (Δ42-54) carrying a 6x His tag at N-terminal	This work
pMSA3	Km^r (pET28a). Expresses the *A. brasilense* GlnZ carrying a 6x His tag at N-terminal	Araujo *et al.*, 2004
pGANAGK	Km^r Expresses the *Azospirillum brasilense* NAGK	This work

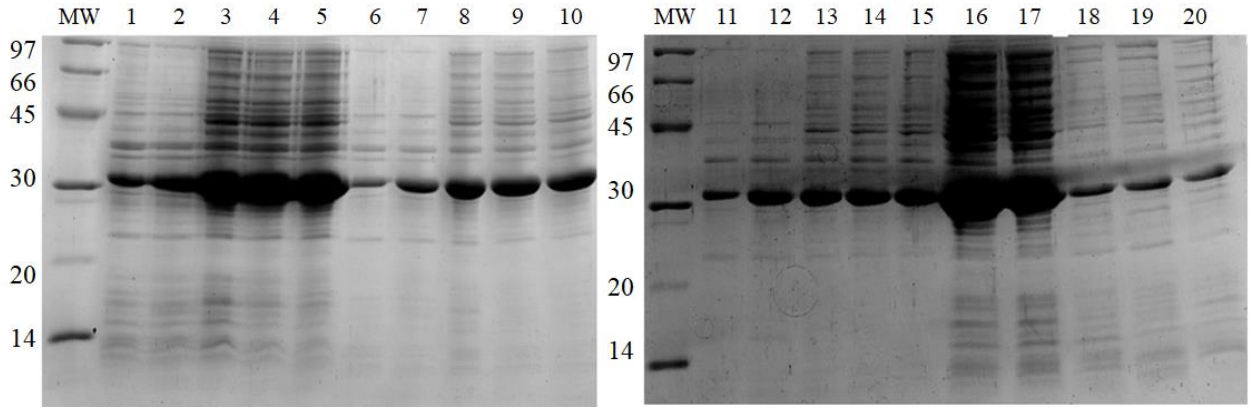

Figure 1 - Binding of NAGK to different matrices. Cell extracts expressing AbNAGK were incubated with different ion exchange resins and submitted to several washes with buffer A (50 mM Tris-HCl pH 7.5, 50 mM NaCl) and eluted with buffer B (50 mM Tris-HCl pH 7.5; 1M NaCl). Lanes 1, 6, 11 – Q FF (GE Healthcare) wash fractions; Lane 16 – Q FF elution fraction. Lanes 2, 6, 12 - DEAE FF (GE Healthcare) wash fractions; Lane 17 – DEAE FF elution fraction. Lanes 3, 8, 13 - CM FF (GE Healthcare) wash fractions, Lane 18 – CM FF elution fraction; Lanes 4, 9, 14 - SP FF (GE Healthcare) wash fractions; Lane 19 – SP FF elution fraction, Lane 5, 10, 15 - Heparin HP (GE Healthcare) wash fractions; Lane 20 – Heparin HP elution fraction.

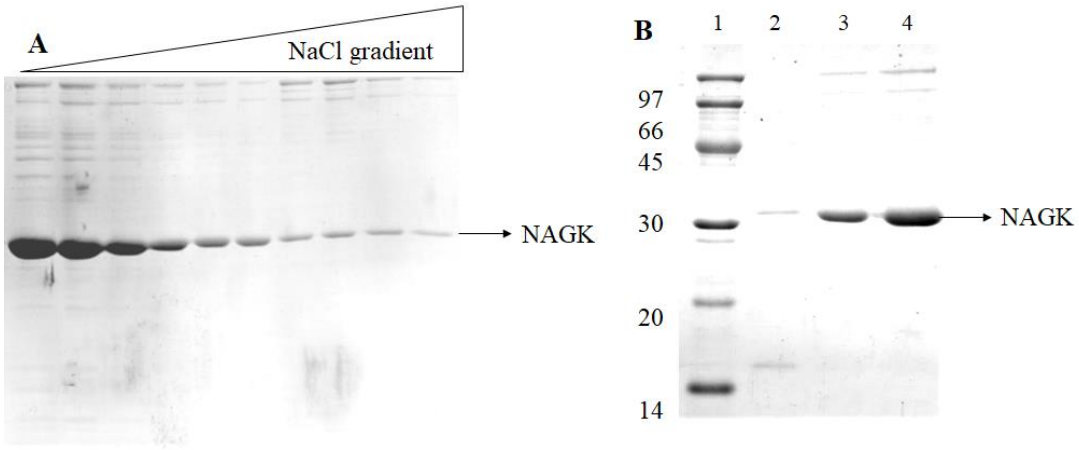

Figure 2 - Analysis of purified *A. brasilense* NAGK. A - Protein purification using Hi-Trap DEAE FF. The protein was eluted with NaCl linear gradient. The samples were analysed on 12,5% SDS-PAGE (Comassie Blue stained). B – SDS-PAGE analysis of AbNAGK: Lane 1, MW Standards in kDa. Lanes 2, 3 and 4: 1, 5 and 10 µg of the purified AbNAGK, respectively. The gel was Coomassie Blue stained.

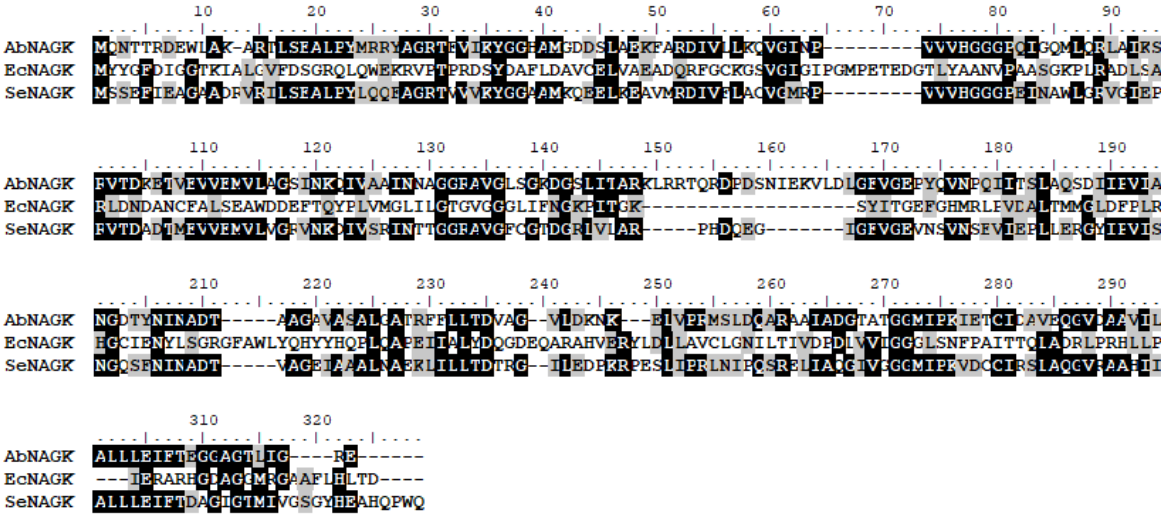

Figure 3 - Sequence alignment of *A. brasilense* NAGK (Uniprot G8AI64), *E. coli* NAGK (WP_101151630.1) and *Synechoccous* sp. NAGK (WP_006455177.1). Sequences were align using Clustal W. Identical residues are shown in black and similar in light gray.

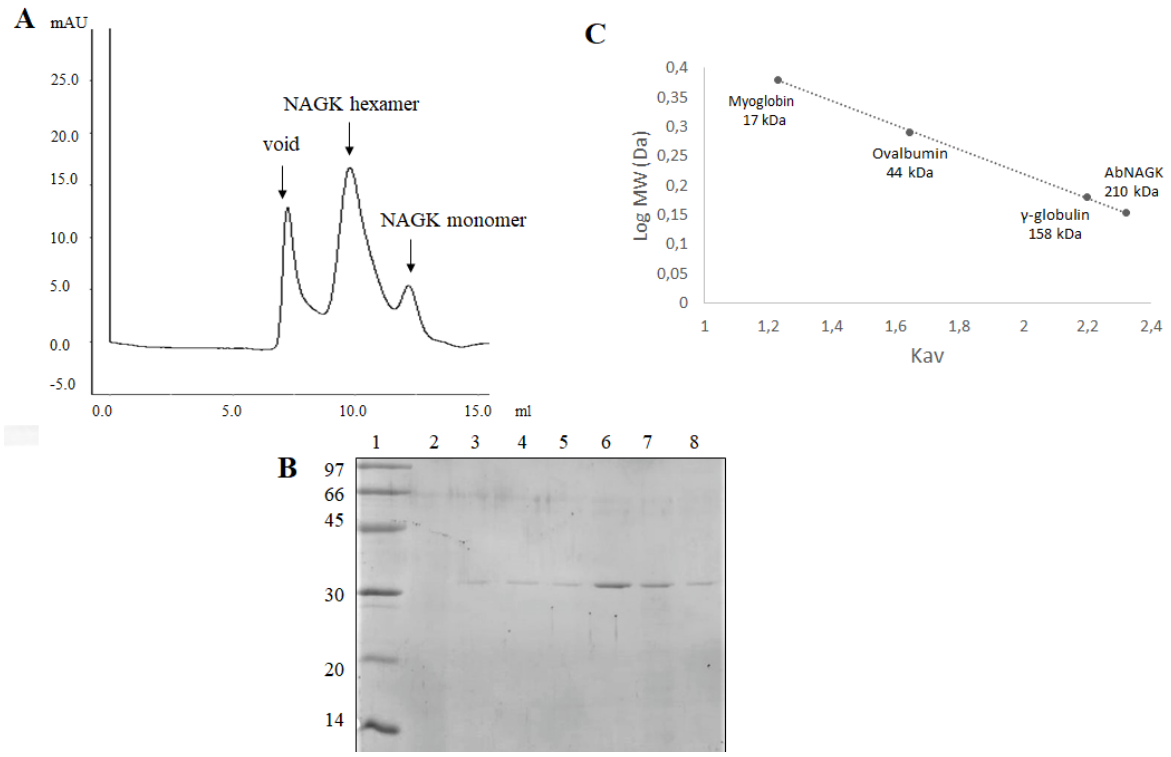

Figure 4 – A- Gel filtration analisys of purified NAGK performed on a Superose 12 10/300 GL column using 50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 100 mM NaCl as buffer. B- SDS-PAGE (Comassie blue satined) of gel filtration fractions. Lane 1- MW standards in kDa. Lanes 2-3 – Fractions of void peak. Lanes 4-6 – Fractions of NAGK hexamer peak. Lanes7-8 – Fractions of NAGK monomer peak. C- Molecular mass standards (BioRad).

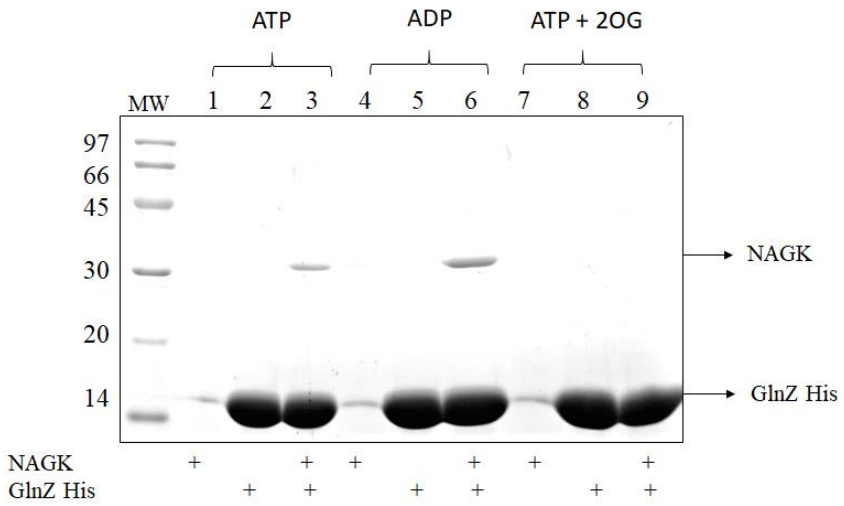

Figure 5 – *In vitro* co-precipitation assay between the GlnZ His and NAGK. GlnZ His (lanes 2, 3, 5, 6, 8 and 9) was bound to Ni^{2+} beads in the presence of the indicated effectors (ATP 1 mM, ADP 1 mM or ATP 1 mM + 2OG 1.5 mM). The beads received purified NAGK as indicated (Lanes 1, 3, 4, 6, 7 and 9). After extensive washes, bound proteins were eluted with imidazol and subjected to SDS-PAGE, the gels were Coomassie blue stained.

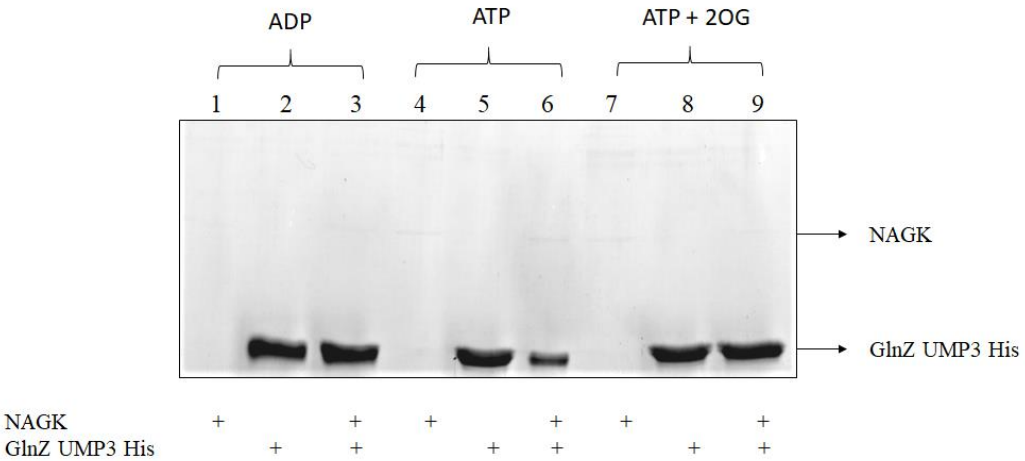

Figure 6 – *In vitro* co-precipitation assay between the fully uridylylated GlnZ-UMP_3 and NAGK. Uridylylated His-GlnZ-UMP_3 (lanes 2, 3, 5, 6, 8 and 9) was bound to Ni^{2+} beads in the presence of the indicated effectors (ATP 1 mM, ADP 1 mM or ATP 1 mM + 2OG 1.5 mM). The beads received purified NAGK as indicated (Lanes 1, 3, 4, 6, 7 and 9). After extensive washes, bound proteins were eluted with imidazol and subjected to SDS-PAGE. The gels were Coomassie blue stained.

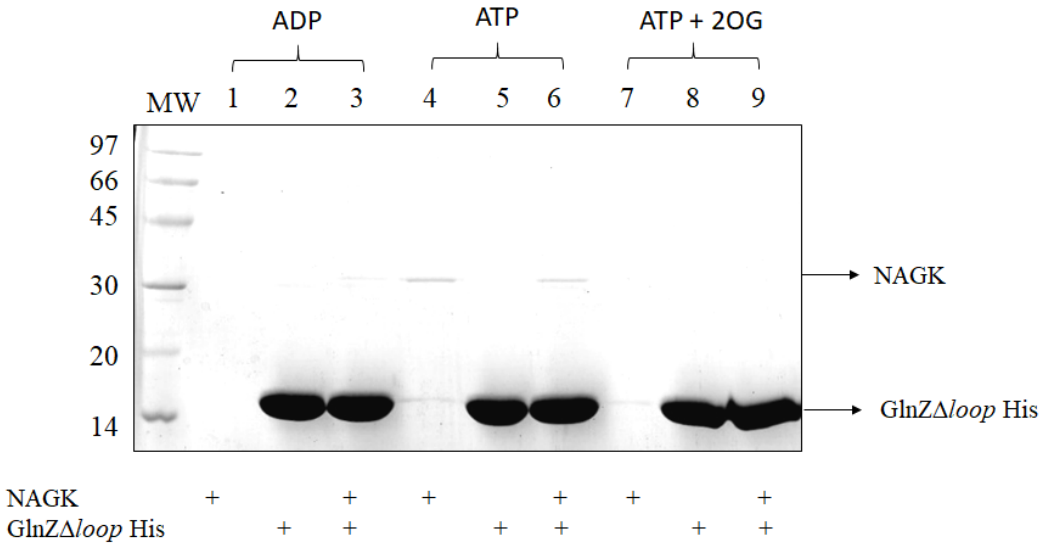

Figure 7 – *In vitro* co-precipitation assay between the GlnZΔ*loop* His and NAGK. GlnZΔ*loop* His (lanes 2, 3, 5, 6, 8 and 9) was bound to Ni^{2+} beads in the presence of the indicated effectors (ATP 1 mM, ADP 1 mM or ATP 1 mM + 2OG 1.5 mM). The beads received purified NAGK as indicated (Lanes 1, 3, 4, 6, 7 and 9). After extensive washes, bound proteins were eluted with imidazol and subjected to SDS-PAGE. The gels were Coomassie blue stained.

5. CONCLUSÕES

- ✓ A enzima málica de *A. brasilense* (AbMaeB1) purificada neste trabalho é $NADP^+$ dependente e requer Mg^{2+} ou Mn^{2+} para sua atividade.
- ✓ AbMaeB1 parece ser octamérica.
- ✓ A atividade de enzima málica de AbMaeB1 é fortemente ativada por KCl e glutamina e inibida por acetil-P e coenzima A.
- ✓ Glutamina também é capaz de ativar enzima málica MaeB de *E. coli* (EcMaeB).
- ✓ A resposta de AbMaeB1 e EcMaeB à glutamina ocorre de forma dose dependente com constantes de ativação de 1,98 e 0,74 mM, respectivamente.
- ✓ AbMaeB1 e EcMaeB possuem o domínio PTA cataliticamente ativos capazes de converter coenzima A e Acetil-fosfato em Acetil-CoA .
- ✓ AbMaeB1 interage *in vitro* com GlnZ na presença de ATP e ADP.
- ✓ O complexo AbMaeB-GlnZ é desestabilizado quando GlnZ está uridililada.
- ✓ O *loop* T não é essencial para a formação do complexo AbMaeB1-GlnZ.
- ✓ GlnZ parece ativar a atividade málica de AbMaeB1.
- ✓ A enzima N-acetil glutamato quinase de *A. brasilense* (AbNAGK) purificada neste trabalho é hexamérica.
- ✓ AbNAGK interage com GlnZ *in vitro* na presença de ATP e ADP.
- ✓ O complexo AbNAGK-GlnZ não ocorre quando GlnZ está uridililada.
- ✓ O *loop* T parece ser essencial para a formação do complexo AbNAGK-GlnZ.
- ✓ Por meio destas novas interações, PII parece estar envolvida na regulação do metabolismo de carbono, biossíntese de NADPH e produção/excreção de acetato, bem como com a biossíntese de arginina em bactérias.

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